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(71) Applicants *(for all designated States except US)*: THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19014 (US). APOLLON, INC. [US/US]; One Great Valley Parkway, Malvern, PA 19355 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KIM, Jong, J. [US/US]; 157 Orchard Drive North, Wales, PA 19454 (US). BAGARAZZI, Mark, L. [US/US]; 769 Mount Vernon Avenue, Haddonfield, NJ 08033 (US). WANG, Bin [CN/CN]; Sinogen Institute, Institute of Zoology, Chinese Academy of Sciences, 19 Zhongguancun Lu, Mail Box 30, Haidian, Beijing 100080 (CN). BOYER, Jean, D. [US/US]; 215 Woodlane Avenue, Haddonfield, NJ 08033 (US). WEINER, David, B. [US/US]; 717 Beacom Lane, Merion Station, PA 19066 (US).

(74) Agents: ELDERKIN, Dianne, B. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris LLP, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).

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(54) Title: IMMUNOTHERAPY AND IMPROVED VACCINES

(57) Abstract

Improved vaccines which include a nucleotide sequence that encodes IL–12, GM–CSF, IL–1, TNF–α, TNF–β, IL–2, IL–4, IL–5, IL–10, IL–15, IL–18, and/or BL–1 operably linked to regulatory elements are disclosed. The improved vaccines include DNA vaccines, recombinant vaccines for delivering foreign antigen and live attenuated vaccines. Methods of immunizing individuals are disclosed. Pharmaceutical compositions comprising nucleic acid molecules that encode one or more immunomodulatory proteins selected from the group consisting of IL–12, GM–CSF, IL–1, TNF–α, TNF–β, IL–2, IL–4, IL–5, IL–10, IL–15, IL–18, and BL–1 are disclosed. An immunomodulatory protein, BL–1, and nucleic acid molecules that encode BL–1 are disclosed. Methods of making and using BL–1 are disclosed.

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IMMUNOTHERAPY AND IMPROVED VACCINES

FIELD OF THE INVENTION

The present invention relates to immunotherapeutic compositions and methods, and to improved protective and therapeutic vaccines and improved methods for prophylactically and/or therapeutically inducing immune responses against antigens.

BACKGROUND OF THE INVENTION

Vaccines are useful to immunize individuals against target antigens such as pathogen antigens or antigens associated with cells involved in human diseases. Antigens associated with cells involved in human diseases include cancer-associated tumor antigens and antigens associated with cells involved in autoimmune diseases.

The overall objective of any immunization strategy is to induce specific immune responses which protect the immunized individual from a given pathogen over his or her lifetime. One major challenge in meeting this goal is that the correlates of protection from an individual pathogen vary from one infectious agent to the next. Therefore, a more clinically effective vaccine should elicit a more specific immune responses against the targeted pathogen. Immunization strategies need to be designed which can be "focused" according to the correlates of protection known for the particular pathogen.

In designing such vaccines, it has been recognized that vaccines which produce the target antigen in the cell of the vaccinated individual are effective in inducing the cellular arm of the immune system. Specifically, live attenuated vaccines, recombinant vaccines which use avirulent vectors and DNA vaccines all lead to the production of antigens in the cell of the vaccinated individual which results induction of the cellular arm of the immune system. On the other hand, sub-unit vaccines which comprise only proteins and killed or inactivated vaccines, which do induce a humoral response, do not induce good cellular immune responses.

A cellular immune response is often necessary to provide protection against pathogen infection and to provide effective immune-mediated therapy for treatment of pathogen infection, cancer or autoimmune diseases. Accordingly, vaccines which produce the target antigen in the cell of the vaccinated individual such as live attenuated vaccines, recombinant vaccines which use avirulent vectors and DNA vaccines are preferred.

Nucleic acid immunization is a new vaccination technique which delivers DNA constructs encoding for a specific immunogen into the host. In addition to DNA vaccine's ability to induce both antigen-specific cellular and humoral immune responses, this technique has the potential to manipulate the immune responses generated through the co-delivery of immunologically important molecules.

While such vaccines are often effective to immunize individuals prophylactically or therapeutically against pathogen infection or human diseases, there is a need for improved vaccines. There is a need for compositions and methods which produce an enhanced immune response.

SUMMARY OF THE INVENTION

The present invention relates to gene constructs which comprise nucleotide sequences that encode immunomodulating proteins which can be administered to

individuals undergoing prophylactic or therapeutic vaccination protocols or therapeutic immunodulation protocols. The immunodulating proteins include human proteins IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and a novel molecule designated BL-1.

The present invention relates to gene constructs that comprise: a nucletide sequence that encodes IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, or BL-1; or nucleotide sequences that encode two or more of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, or BL-1. It is intended that gene constructs can contain multiple copies of the same nucleotide sequence.

The present invention relates to methods of vaccinating an individual by administering a vaccine composition to introduce an immunogen to the individual in combination with the introduction of the gene constructs which comprise nucleotide sequence(s) that encodes one or more immunomodulating proteins which results in an enhanced and/or more desirable immune response. Moreover, the present invention relates to methods of modulating the immune response of an individual by administering a gene construct which comprises nucleotide sequence(s) that encode one or more immunomodulating proteins. The modulation of the immune response may be a step in a vaccination protocol in which the patients immune response is switched from a primarily Th1 to Th2 response or vice-versa by first co-administering a vaccine composition with an immunomodulating protein that favors one form of immune response and boosting the individual by co-administering the vaccine composition with an immunomodulating protein that favors the other form of immune response.

The vaccine compositions are preferably plasmids which are directly introduced into the individual. Similarly, the gene construct that comprises nucleotide sequence(s) that encode one or more immunomodulating proteins is preferably a plasmid.

The present invention relates to a plasmid which comprises nucleotide sequences that encode human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a nucleotide sequence that encodes an immunogenic target antigen operably linked to regulatory elements necessary for expression in eukaryotic cells. In some preferred embodiments, the immunogenic target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases. In some embodiments, the plasmid comprises a nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells. The single chain IL-12 protein is a single protein which is encoded by a single coding sequence and which includes a linker connecting the two subunits. The linker is sufficiently large enough and flexible enough to allow the single protein to fold into the biologically active conformation assumed by the functional, native two-subunit IL-12 protein.

The present invention relates to a method of inducing, in an individual, an immune response against an antigen comprising the step of administering to an individual, a plasmid which comprises a nucleotide sequence that encode human IL-12 protein operably linked to regulatory elements necessary for expression in cells of the individual, and a nucleotide sequence that encodes a target antigen operably linked to regulatory elements necessary for expression in cells of the individual. In some preferred embodiments, the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases. In preferred embodiments, the immune response that is induced against the target antigen provides a therapeutic benefit with respect to infections, diseases, disorders and conditions associated with the proteins to which the anti-antigen immune response is directed and/or a protective immune response is induced against pathogens or cells having proteins that cross react to the immune response

generated against the antigen. In some embodiments, the human IL-12 protein is a single chain IL-12 protein.

The present invention relates to a composition which comprises a plurality of plasmids which collectively comprise nucleotide sequences encoding both subunits of human IL-12 and a target antigen, each coding sequence being operably linked to regulatory elements necessary for gene expression. In some embodiments, the composition includes two plasmids: a first plasmid which comprises nucleotide sequences that encode IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a second plasmid which comprises a nucleotide sequence that encodes an immunogenic target antigen operably linked to regulatory elements necessary for expression in eukaryotic cells. In some embodiments, one plasmid contains a nucleotide sequence that encodes the immunogenic target protein and one subunit of human IL-12, and a second plasmid contains a nucleotide sequence that encodes the other subunit of human IL-12. In some embodiments, three different plasmids are provided: one that contains a nucleotide sequence that encodes the immunogenic target protein, one that encodes the p35 subunit of human IL-12 and one that encodes the p40 subunit of human IL-12. In some embodiments, the composition includes two plasmids: a first plasmid which comprises a nucleotide sequence that encodes a single chain IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a second plasmid which comprises a nucleotide sequence that encodes an immunogenic target antigen operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of inducing, in an individual, an immune response against an antigen comprising the step of administering to an individual, a composition which comprises a plurality of plasmids which collectively which collectively comprise nucleotide sequences encoding both subunits of human IL-12 and a target antigen, each coding sequence being operably linked to regulatory

elements necessary for gene expression. In some embodiments, two or three plasmids as described above which together comprise a nucleotide sequences that encode both subunits of human IL-12 protein and an immunogenic target protein, the nucleotide sequences that encode protein being operably linked to regulatory elements necessary for expression in cells of the individual. In preferred embodiments, the immune response induced by the target antigen cross reacts to a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases. The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease. In preferred embodiments, the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases. In some embodiments, human IL-12 is a single chain IL-12 protein.

The present invention relates to a plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 and IL-10 operably linked to regulatory elements necessary for expression in eukaryotic cells and a nucleotide sequence that encodes an immunogenic target antigen operably linked to regulatory elements necessary for expression in eukaryotic cells. In some preferred embodiments, the immunogenic target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a method of inducing, in an individual, an immune response against an antigen comprising the step of administering to an individual, a plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in cells of the individual, and a nucleotide sequence that encodes a target antigen operably linked to regulatory elements necessary for expression in cells of the individual. In some preferred

embodiments, the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases. In preferred embodiments, the immune response that is induced against the target antigen provides a therapeutic benefit with respect to infections, diseases, disorders and conditions associated with the proteins to which the anti-antigen immune response is directed and/or a protective immune response is induced against pathogens or cells having proteins that cross react to the immune response generated against the antigen.

The present invention relates to a composition which comprises a plurality of plasmids which includes two plasmids: a first plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a second plasmid which comprises a nucleotide sequence that encodes an immunogenic target antigen operably linked to regulatory elements necessary for expression in eukaryotic cells. In some embodiments, the composition comprises three plamids including a third plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in eukaryotic cells. In some embodiments, the composition comprises four plamids including a third plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a fourth plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of inducing, in an individual, an immune response against an

antigen comprising the step of administering to an individual, a composition which comprises a plurality of plasmids which includes two plasmids: a first plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for gene expression and a second plasmid which comprises a nucleotide sequence that encodes an immunogenic target antigen operably linked to regulatory elements necessary for expression. In preferred embodiments, the immune response induced by the target antigen cross reacts to a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases. The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease. In preferred embodiments, the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases. In some embodiments, the method comprises administering a composition that comprises three plamids including a third plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in eukaryotic cells. In some embodiments, the method comprises administering a composition that comprises four plamids including a third plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a fourth plasmid which comprises nucleotide sequence(s) that encode one or more of human GM-CSF, IL-1α, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 or IL-10 protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to an improved recombinant vaccine vector which comprises nucleotide

sequence(s) that encode one or more of human IL-12, GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5, IL-10 of BL-1 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a nucleotide sequence that encodes a target antigen operably linked to regulatory elements necessary for expression in eukaryotic cells. In some embodiments, genes encoding human IL-12 protein encode IL-12 as a single chain protein. In preferred embodiments, the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease comprising the step of administering to an individual, a recombinant vaccine vector which comprises nucleotide sequence(s) that encode one or more of human IL-12, GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5, IL-10 of BL-1 protein operably linked to regulatory elements necessary for expression in cells of the individual, and a nucleotide sequence that encodes a target antigen operably linked to regulatory elements necessary for expression in cells of the individual, wherein the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to an improved live, attenuated vaccine which comprises nucleotide sequence(s) that encode one or more of human IL-12, GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5, IL-10 of BL-1 protein or single chain IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease comprising the step of administering to an individual, an attenuated vaccine which comprises nucleotide sequence(s) that encode one or more of human IL-12, GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5, IL-10

of BL-1 or single chain IL-12 protein operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to a plasmid which comprises nucleotide sequence(s) that encode one or more of human IL-12, GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5, IL-10 of BL-1 protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a pair of plasmids which one plasmid comprises a nucleotide sequence that encodes human IL-12 protein p35 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells and the other plasmid comprises a nucleotide sequence that encodes human IL-12 protein p40 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a plasmid which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule.

The present invention relates to a pharmaceutical composition which comprises a plasmid which comprises nucleotide sequence(s) that encode one or more of IL-12, GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5, IL-10 of BL-1 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a pharmaceutically acceptable carrier or diluent.

The present invention relates to a pharmaceutical composition which comprises a pair of plasmids which one plasmid comprises a nucleotide sequence that encodes human IL-12 protein p35 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells and the other plasmid comprises a nucleotide sequence that encodes human IL-

12 protein p40 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells and a pharmaceutically acceptable carrier or diluent.

The present invention relates to a pharmaceutical composition which comprises a plasmid which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule and a pharmaceutically acceptable carrier or diluent.

The present invention relates to a method of treating an individual who is suffering from an allergic reaction, a pathogen infection, cancer or an autoimmune disease comprising the step of administering to an individual, a plasmid which comprises nucleotide sequences that encode IL-12 protein operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to a method of treating an individual who is suffering from an allergic reaction, a pathogen infection, cancer or an autoimmune disease comprising the step of administering to an individual, a pair of plasmids which one plasmid comprises a nucleotide sequence that encodes human IL-12 protein p35 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells and the other plasmid comprises a nucleotide sequence that encodes human IL-12 protein p40 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of treating an individual who is suffering from an allergic reaction, a pathogen infection, cancer or an autoimmune disease comprising the step of administering to an individual, a plasmid which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to

regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule.

The present invention relates to a method of enhancing or driving an immune response in an individual toward a Th1 type immune response comprising the step of administering to an individual, a plasmid which comprises nucleotide sequences that encode human IL-12 protein operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to a method of enhancing a TH1 type immune response in an individual or driving an immune response in an individual toward a Th1 type immune response comprising the step of administering to an individual, a pair of plasmids which one plasmid comprises a nucleotide sequence that encodes human IL-12 protein p35 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells and the other plasmid comprises a nucleotide sequence that encodes human IL-12 protein p40 subunit operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of enhancing a TH1 type immune response in an individual or driving an immune response in an individual toward a Th1 type immune response comprising the step of administering to an individual, a plasmid which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule.

The present invention relates to a recombinant vector which comprises nucleotide sequences that encode human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a recombinant vector which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule.

The present invention relates to a pharmaceutical composition which comprises a recombinant vector which comprises nucleotide sequence(s) that encode one or more of human IL-12, GM-CSF, IL-1α, TNF-α and TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5, IL-10 of BL-1 protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a pharmaceutically acceptable carrier or diluent.

The present invention relates to a pharmaceutical composition which comprises a recombinant vector which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule and a pharmaceutically acceptable carrier or diluent.

The present invention relates to a method of treating an individual who is suffering from an allergic reaction, a pathogen infection, cancer or an autoimmune disease comprising the step of administering to an individual, a recombinant vector which comprises nucleotide sequences that encode human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of treating an individual who is suffering from an allergic reaction, a pathogen infection, cancer or an autoimmune disease comprising the step of administering to an individual, a recombinant vector which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule.

The present invention relates to a method of enhancing a TH1 type immune response in an individual or driving an immune response in an individual toward a Th1 type immune response comprising the step of administering to an individual, a recombinant vector which comprises nucleotide sequences that encode human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of enhancing a TH1 type immune response in an individual or driving an immune response in an individual toward a Th1 type immune response comprising the step of administering to an individual, a recombinant vector which comprises a single nucleotide sequence that encodes a single chain human IL-12 protein operably linked to regulatory elements necessary for expression in eukaryotic cells wherein the single chain human IL-12 protein is a single protein in which the p35 and p40 subunits are connected to each other by a linker sequences wherein when expressed the single chain protein can form a biologically active IL-12 molecule.

The present invention provides compositions that comprise nucleic acid molecules that encode one or more human proinflammatory cytokines (IL-1α, TNF-α and TNF-β), Th1 cytokines (IL-2, IL-15, and IL-18), and Th2 cytokines (IL-4, IL-5 and IL-10) protein as a primary agent and to methods of

using the same to drive and direct an immune response by administering such nucleic acid molecules to an individual. The present invention relates to methods of enhancing antigen-specific humoral response by the co-delivery of IL-5 or IL-18 with vaccines that introduce target immunogens such as DNA vaccine constructs. The present invention relates to methods of increasing antigen-specific T helper cell proliferation by co-delivery of IL-2, IL-5, IL-10, IL-18, TNF-α or TNF-β with vaccines that introduce target immunogens such as DNA vaccine constructs. The present invention relates to methods of enhancing the cytotoxic response with the co-administration of TNF-α or IL-15 genes with vaccines that introduce target immunogens such as DNA vaccine constructs.

The present invention provides compositions that comprise nucleic acid molecules that encode human GM-CSF, and to method of inducting and regulating immune responses by delivering or co-delivering gene constructs that encode GM-CSF. The present invention relates to methods of enhancing antigen-specific antibody and T helper cell proliferation responses by co-injection of GM-CSF genes with DNA vaccine constructs.

The present invention relates to substantially pure BL1 and immunomodulating fragments thereof.

The present invention relates to isolated nucleic acid molecules that encode BL1 and immunomodulating fragment thereof.

The present invention relates to nucleic acid probes and primers specifically directed to nucleic acid molecules that encode BL1, or immunomodulating fragments thereof.

The present invention relates to oligonucleotide molecules that consist of a nucleotide sequence complementary to a specific portion of the nucleotide sequence that encodes BL1.

The present invention relates to vectors comprising nucleic acid molecules encoding BL1 or immunomodulating fragments thereof.

The present invention relates to recombinant expression vectors that comprise nucleic acid sequences that encode BL1 or immunomodulating fragments thereof.

The present invention relates to host cells that comprise recombinant expression vectors which include nucleic acid sequences that encode BL1 or immunomodulating fragments thereof. The present invention relates to genetic therapy vectors comprising nucleic acid molecules encoding BL1 or immunomodulating fragments thereof.

The present invention relates to isolated antibody which binds to a specific epitope on BL1.

The present invention is related to methods of making BL1 and immunomodulating fragments thereof.

The present invention is related to methods of modulating an immune response in an individual comprising administering to the individual BL1 protein or an immunomodulating fragment thereof, or a vector which comprises a nucleotide sequence that encodes BL1 protein or an immunomodulating fragment thereof. According to aspects of the invention, the vector which comprises the BL1 coding sequence is sufficient to modulate the immune response.

The present invention is related to methods of enhancing and directing an immune response in an individual comprising administering to the individual a vaccine composition for delivery of an immunogen and a BL1 protein or an immunomodulating fragment thereof, or a vector which comprises a nucleotide sequence that encodes BL1 protein or an immunomodulating fragment thereof. According to aspects of the invention, the vector which comprises the BL1 coding sequence is sufficient to modulate the immune response.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B shows data from Example 4. In Figure 1A, 50 μg of respective cDNA expression cassettes were administered intramuscularly at day 0. At 14 days post-immunization, spleens harvested from all immunized animals were weighed. The negative control animals were immunized.

the spleens from the mice injected with Gag/Pol alone and IL-12 alone weighed similar to those from the unimmunized control mice (about 100 mg). However, the spleens from mice injected with Gag/Pol+IL-12 genes weighed almost three times as much as the control spleens. In contrast, Gag/Pol+GM-CSF immunized mouse spleens were not enlarged. In Figure 1B, the white blood cells were prepared and purified from each spleen. Corresponding directly to their spleen weight difference, the number of cells from the Gag/Pol+IL-12 immunized spleens were more than three the number derived from the control spleens. Gag/Pol+GM-CSF immunized mouse spleens did not have any significant increase in the number of lymphocytes above the control spleen cell number.

In Figure 2, 50 μg of respective cDNA expression cassettes were administered intramuscularly at day 0. At 14 days post-immunization, spleens harvested and were photographed. The visual size of the spleens corresponded directly to the weights where the immunogen+IL-12 vaccinated spleens were significantly larger than the unimmunized control spleens. Groups: (-) unimmunized; IL-12 immunized; Envelop+IL-12 immunized; Gag/Pol+IL-12 immunized.

In Figure 3, co-administration of each chain of IL-12 was performed. 50μg of each plasmid was used. Both p35 and p40 chains as well as Gag/Pol were necessary for spleen enlargement.

In Figure 4, 50μg of respective cDNA expression cassettes were administered intramuscularly at day 0. Antisera from immunized mice were collected and analyzed for specific antibody responses against HIV-1 antigens by ELISA. The ELISA results from the samples collected at day 28 is shown. At 1:100 dilution, sera from the group immunized with Envelop+GM-CSF showed antibody response against HIV-1 gp120 protein which was greater than those of the group immunized with Envelop only. On the other hand, the group immunized with Envelop+IL-12 showed a significantly less humoral response over the same period.

In Figure 5, activation and proliferation of T helper lymphocytes play a critical role in inducing both humoral immune response via expansion of antigen-activated B cells and cellular immune response via expansion of CD8+ cytotoxic T cells. 50 μg of respective cDNA expression cassettes were administered intramuscularly at day 0. The harvested spleen cells were tested for T cell proliferation. Recombinant p55 protein 20 μg/ml was plated in each well to stimulate proliferation of T cells. 10 μg/ml of lectin PHA was used as a polyclonal stimulator positive control Stimulation index is the level of radioactivity detected from the cells stimulated with specific protein divided by the level detected from the cells in media. The stimulation index of PHA stimulated control was 58.8.

In Figure 6, 50 μg of respective cDNA expression cassettes were administered intramuscularly at day 0. CTL assay without in vitro stimulation as conducted using the cells prepared from harvested spleens. The control group immunized with only IL-12 gene cassette resulted in no specific lysis of target cells above the background level. In addition, low level (3%) of specific lysis was observed with Gag/Pol only immunization at the 50:1 effector:target ratio. In contrast, 62% specific lysis was seen with Gag/Pol+IL-12 co-administration samples at the 50:1 Effector:Target ratio and titered out to 9% at the 12.5:1 Effector:Target ratio. Those immunized with Gag/Pol+GM-CSF plasmids resulted in no detectable CTL activity. The same CTL assay conducted against targets prepared with irrelevant antigen-expressing vaccinia did not result in any significant CTL response.

In Figure 7, 50μg of respective cDNA expression cassettes were administered intramuscularly at day 0. CTL assays without in vitro stimulation was conducted using the cells prepared from harvested spleens. At 50:1 Effector:Target ratio, the group immunized with Envelop only and Envelop+GM-CSF resulted in low levels of specific CTL at 4% and 1%, respectively. On the other hand, a dramatic

enhancement of CTL activity was seen from the Envelop+IL-12 group at 59%. The same CTL assay conducted against targets prepared with irrelevant antigen-expressing vaccinia did not result in any significant CTL response.

Figures 8A, 8B and 8C show plasmids useful in the invention. Figure 8A shows a plasmid that includes a coding sequence for IL-12 as a single chain protein. Figures 8B and 8C show plasmids each include two coding sequences for each of the two subunits.

Figure 9 shows Table 3.

As shown in Figure 10, each cytokine gene was cloned into expression plasmids under the control of a CMV promoter and was transfected *in vitro* into RD cells. Expression of each cytokine was verified using either immunoprecipitation or cytokine ELISA.

Figures 11A and 11B show results from experiments determining MHC Class I-Restricted CTLs. As shown in Figure 11A, two weeks after the first DNA co-injection with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells prepared with envelope-specific peptide (RIHIGPGRAFYTTKN) which has been reported to be MHC class I-restricted in balb/c mice. As shown in Figure 11B, two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response. A CTL assay was performed with the removal of CD8+ T cells by complement lysis. Effector cells were prepared as described with the presence of CD8+ T cells (top) and the removal of CD8+ T cells (bottom). These experiments have been repeated two times with similar results.

Figure 12 show results from experiments evaluating direct antigen-specific CTL (without *in vitro* stimulation of effectors). Two weeks after the first DNA co-injection with

pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vMN462) and non-specific vaccinia (vSC8). These experiments have been repeated with similar results.

Figure 13 shows a summary of the each cytokine co-administration effects on antibody (y-axis), T helper (x-axis), and cytotoxic T lymphocyte responses (z-axis). Each cytokine is plotted on the 3-D axis according to its effects on the three modes of immune response.

Figure 14 shows the nucleotide and predicted amino acid sequences of BL1.

Figure 15 shows the ligation of BL1 into PCR3 eukaryotic expression vector as well as the vector pBBKan.

Figure 16 shows results of ELISA assays comparing anti-HIV antigen responses directed at the HIV antigen Nef with and without con-administration of BL1.

Figure 17A, 17B, 17C and 17D shows results of assays comparing anti-HIV antigen immune responses directed at the HIV antigen Gag/Pol with and without co-administration of BL1.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "immunomodulating protein" is meant to refer to one of human IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-15, IL-18, IL-4, IL-5 and IL-10, and a novel molecule designated BL-1.

As used herein, the term "IL-12 genetic construct" is meant to refer to plasmids which comprise coding sequences that encode one or both human IL-12 protein subunits and/or the immunogenic target protein, the coding sequences being operably linked to regulatory elements required for expression of the coding sequences in eukaryotic cells. Regulatory elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as

a Kozak region, may also be included in the genetic construct. Initiation and termination signals are required regulatory elements which are often considered part of the coding sequence. The coding sequences of genetic constructs of the invention include functional initiation and termination signals.

As used herein, the term "desired IL-12 protein" is meant to refer to one or both human IL-12 subunits including single chain IL-12 proteins in which the two subunits are encoded by a single coding sequence and expressed as a single protein having a linker sequences connecting the two subunits.

As used herein, the term "desired protein" is meant to refer to the immunogenic target protein encoded by the coding sequence of a vaccine that comprises a nucleic acid molecule that encodes an immunogenic target protein.

As used herein, the term "single chain protein" and "single chain IL-12 protein" is meant to refer to a single protein in which the IL-12 p35 and p40 subunits are connected to each other by an amino acid linker that is sufficiently long and flexible enough to allow the single protein to allow the two subunit portions to interact with each other and assume the conformation of the biologically active complex that is IL-12. Single chain IL-12 functions essentially identical as IL-12 made up of p35 and p40. The present invention is meant to include the use of single chain IL-12 in all places where IL-12 is used. The single protein is encoded by a single nucleotide sequence.

Interleukin-12 (IL-12), a heterodimeric cytokine produced primarily by macrophages and B cells. IL-12 is composed of two different subunits which are designated p35 and p40 (Podlaski, F.J. *et al.* (1991) *Arch. Biochem. Biophys.* 294(1):230-237, which is incorporated herein by reference).

Different immune responses involve T cell populations. Specifically, there are two distinct types of T cells, Type 1 T-helper cells (Th1) and Type 2 T-helper cells (Th2), which differ from each other, among other things, in their production of cytokines. IL-12 has been found to play

a critical role in Th1 immune response mainly by inducing production of Th1-associated cytokine interferon-gamma (IFN-gamma). It activates natural killer (NK) and T cells through induction and release of various cytokines including IFN-gamma.

Aspects of the present invention relates to the use of nucleic acid molecules that encode human IL-12 protein as an immunomodulator. The nucleic acid molecules that encode human IL-12 protein may be delivered as the primary active agent, i.e. as a gene therapeutic, or as part of or in conjunction with vaccine compositions such as vaccines which comprise nucleic acid molecules that encode immunogenic target proteins.

With regard to the use of nucleic acid molecules that encode human IL-12 protein as a primary agent, the present invention provides compositions and methods for driving an immune response toward or enhancing a Th1 immune response by administering nucleic acid molecules that encode human IL-12 protein to an individual. According to some aspects of the invention, individuals suffering from allergy disorders, pathogen infections, cancer or autoimmune diseases can be treated by administering to such individuals, nucleic acid molecules that include nucleotide sequences that encode human IL-12 operably linked to regulatory elements such that nucleic acid molecules are expressed in cells of the individual. The nucleic acid molecules are taken up by the cells and the nucleotide sequence that encodes human IL-12 is expressed. The human IL-12 thus produced by the cell is biologically active and its activity results in the induction and/or enhancement of the immune response generated by the individual. In some preferred embodiments, the nucleic acid molecule that encodes human IL-12 protein is a plasmid.

Aspects of the invention include the use of nucleic acid molecules that encode granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF is a hematopoietic growth factor which stimulates neutrophil, monocyte/macrophage, and eosinophil colony formation. It also induces proliferation

and differentiation of erythroid and megakaryocyte progenitor cells. GM-CFS also increases the antibody-dependent cell-mediated cytotoxicity of neutrophiles, eosinophils, and macrophages but has not been reported to have a direct role in the generation of CTL response *in vivo*. The present invention provides compositions and methods for driving an immune response by administering gene constructs that include nucleotide sequences that encode GM-CSF to an individual. The nucleic acid molecules are taken up by the cells and the nucleotide sequence that encodes GM-CSF is expressed and thus produced by the cell. The GM-CSF is biologically active and its activity results in the induction and/or enhancement of the immune response generated by the individual. In some preferred embodiments, the nucleic acid molecule that encodes GM-CSF is a plasmid.

Aspects of the invention include the use of nucleic acid molecules that encode human proinflammatory cytokines (IL-1α, TNF-α and TNF-β), Th1 cytokines (IL-2, IL-15, and IL-18), and Th2 cytokines (IL-4, IL-5 and IL-10) protein as a primary agent. The present invention provides compositions and methods for driving an immune response toward or enhancing an immune response by administering nucleic acid molecules that encode human proinflammatory cytokines (IL-1α, TNF-α and TNF-β), Th1 cytokines (IL-2, IL-15, and IL-18), and Th2 cytokines (IL-4, IL-5 and IL-10) to an individual. According to some aspects of the invention, individuals are treated by administering nucleic acid molecules that include nucleotide sequences that encode human proinflammatory cytokines (IL-1α, TNF-α and TNF-β), Th1 cytokines (IL-2, IL-15, and IL-18), and Th2 cytokines (IL-4, IL-5 and IL-10) operably linked to regulatory elements such that nucleic acid molecules are expressed in cells of the individual. The nucleic acid molecules are taken up by the cells and the nucleotide sequence that encodes the protein is expressed. The human protein thus produced by the cell is biologically active and its activity results in the induction and/or enhancement of

the immune response generated by the individual. In some preferred embodiments, the nucleic acid molecule is a plasmid.

With regard to the use of nucleic acid molecules that encode human IL-12 protein as part of or in conjunction with nucleic acid molecules that encode immunogenic target protein, i.e. as part of a vaccine to induce an immune response against the immunogenic protein, the nucleic acid molecules that encode human IL-12 protein may be a component of a vaccine that includes a nucleic acid molecules that encodes the immunogenic target protein, a component of a vaccine that includes immunogenic target, or a separate composition that is co-administered with either a vaccine that includes a nucleic acid molecules that encodes the immunogenic target protein or vaccine that includes immunogenic target. In some preferred embodiments, the nucleic acid molecule that encodes human IL-12 protein is a plasmid and the vaccine is a DNA vaccine that comprises a plasmid which encodes the immunogenic target protein. In some preferred embodiments, the DNA vaccine comprises a plasmid which encodes the immunogenic target protein and human IL-12 protein.

IL-12 is described in published PCT application WO 90/05147 published May 17, 1990 which is incorporated herein by reference. Wolf, S.F. *et al.* 1991 *J.Immunol.* **146**(9):3074-3081, which is incorporated herein by reference, discloses the nucleotide sequence of cDNA that encodes IL-12 as well as the predicted amino acid sequence of the IL-12 protein. Native human IL-12 protein consists of two subunits, p35 and p40. The two subunits form a heterodimeric complex that is biologically active.

According to some embodiments of the invention, the nucleotide sequences that encode each subunit of IL-12 are on a single plasmid, non-plasmid nucleic acid molecule, or viral or microbial genome, wherein the nucleotide sequence encoding each subunit being under the control of its own set of regulatory elements. In some preferred embodiments, coding sequences for both subunits of IL-12 are on a single plasmid; each coding sequence being operably linked to its own set of

regulatory elements. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for both subunits. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on a separate plasmid from a plasmid which contains the coding sequences for both subunits and the two plasmids are delivered to an individual.

According to some embodiments of the invention, the nucleotide sequence that encodes the p35 subunit is on a first plasmid and the nucleotide sequence that encodes the p40 subunit is on a second plasmid and the two plasmids are co-administered to the same site on an individual. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for the p35 subunit. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for the p40 subunit. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on a separate plasmid from either plasmid which contains the coding sequences for respective subunits and the three plasmids are delivered to an individual.

IL-12 protein, and the nucleotide sequence encoding it, may be modified so that the two subunits are encoded by a single nucleotide sequence and expressed as a single chain (fusion) protein molecule. According to the invention, a linker amino acid sequence is provided which essentially connects the two subunits but which is flexible enough so that a biologically active protein can form by the complexing of different portions of the single chain protein. Figure 8A shows an example of a single chain protein in which the coding sequence for the single chain protein is under the control of a human cytomegalovirus promoter. The coding sequence of the single chain protein includes, from 5' to 3', the coding sequence of the p35 subunit, a coding sequence for a linker

and the coding sequence of the p40 subunit as a single coding sequence. It is contemplated that in an alternative arrangement, the coding sequence of the single chain protein includes the coding sequence of the p40 subunit, a coding sequence for a linker and the coding sequence of the p35 subunit as a single coding sequence. The linker must be long enough and flexible enough to allow the two parts of the single protein to assume positions relative to each other such that a biologically active complex is formed.

According to some embodiments of the invention, the nucleotide sequences that encode single chain IL-12 proteins in which the two subunits are joined by a linker to form a single protein are incorporated into a plasmid, non-plasmid nucleic acid molecule, or viral or microbial genome, and operably linked to regulatory elements necessary for expression in eukaryotic cells. In preferred embodiments, the nucleotide sequences that encode the single chain proteins in which the two subunits are joined by a linker to form a single protein are incorporated into a plasmid. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on the same plasmid as the coding sequences for the single chain IL-12 protein. In some embodiments, the coding sequence for a target immunogenic protein, operably linked to regulatory elements, is on a separate plasmid from the plasmid which contains the coding sequences for the single chain protein and the two plasmids are delivered to an individual.

According to aspects of the present invention relate to improved methods and compositions for vaccination, particularly DNA vaccination in which DNA that encodes target immunogens is administered into the individual in whom the DNA is taken up and expressed and an immune response is generated against the immunogen. According to aspects of the invention, DNA that encodes immunomodulating proteins is co-delivered to the individual and the expression of such DNA produces the immunomodulating protein which controls the magnitude and direction of the immune response in order to induce specific

immune responses to tailor the immunization more closely to the correlates of protection which vary from disease to disease.

It has been discovered that the co-production of IL-12 protein in cells of a vaccinated individual that are expressing target antigens results in an surprisingly enhanced immune response against the target antigen. By providing an expressible form of nucleotide sequence that encodes IL-12 protein, vaccines which function by expressing target antigen in the cells of the vaccinated individual, such as DNA vaccines, recombinant vector vaccines and attenuated vaccines, the vaccines are improved.

The co-production of IL-12 in cells producing antigens results in enhanced cellular immunity against the antigen. Accordingly, the present invention provides improved vaccines by providing a nucleotide sequence that encodes IL-12 operably linked to necessary regulatory sequences for expression in vaccinees as part of vaccines such as DNA vaccines, avirulent recombinant vector vaccines and live attenuated vaccines.

The present invention provides induction and regulation of immune responses from the co-delivery of gene constructs that encode GM-CSF. Co-injection of GM-CSF genes with DNA vaccine constructs enhances antigen-specific antibody and T helper cell proliferation responses.

The present invention provides induction and regulation of immune responses from the co-delivery of gene constructs that encode proinflammatory cytokines (IL-1α, TNF-α and TNF-β), Th1 cytokines (IL-2, IL-15, and IL-18), and Th2 cytokines (IL-4, IL-5 and IL-10).

Some aspects of the present invention provide a significant enhancement of antigen-specific humoral response by the co-delivery of IL-5 or IL-18.

Some aspects of the present invention provide an increase in antigen-specific T helper cell proliferation by co-delivery of IL-2 IL-5, IL-10, IL-18, TNF-α or TNF-β.

Some aspects of the present invention provide an enhancement of the cytotoxic response with the co-administration of TNF-α or IL-15 genes.

Thus, in a case where T cell mediated response is paramount, but the humoral response may not be needed or even be harmful, IL-12 genes are preferred as the immune modulator to be co-delivered with a specific DNA immunogen. On the other hand, for building vaccines to target extracellular bacteria, for example, IL-4, IL-5 or IL-10 genes could be co-injected. Furthermore, in cases where both CD4+ T helper cells and antibodies play more important roles in protection, GM-CSF as well as IL-2 could be co-delivered. Lastly, in cases where all three arms of immune responses are critical, TNF-α could be co-injected to give a combined enhancement of antibody, T helper cell, and CTL responses.

The nucleotide and amino acid sequences of human IL-1α are well known and set forth in Telford, et al. (1986) Nucl. Acids Res. 14:9955-9963, Furutani, et al. (1985) Nucl. Acids Res. 14:3167-3179, March, et al. (1985) Nature 315:641-647, and accession code Swissprot PO1583, which are each incorporated herein by reference.

The nucleotide and amino acid sequences of human IL-2 are well known and set forth in Holbrook, et al. (1984) Proc. Natl. Acad. Sci. USA 81:1634-1638, Fujita, et al. (1983) Proc. Natl. Acad. Sci. USA 80:7437-7441, Fuse, et al. (1984) Nucl. Acids Res. 12:9323-9331, Taniguchi, et al. (1983) Nature 302:305-310, Maeda, et al. (1983) Biochem. Biophys. Res. Comm. 115:1040-1047, Devos, et al. (1983) Nucl. Acids Res. 11:4307-4323, and accession code Swissprot PO1585, which are each incorporated herein by reference.

The nucleotide and amino acid sequences of human IL-4 are well known and set forth in Arai, et al. (1989) J. Immunol. 142:274-282, Otsuka, et al. (1987) Nucl. Acids Res. 15:333-344, Yokota, et al. (1986) Proc. Natl. Acad. Sci. USA 83:5894-5898, Noma, et al. (1984) Nature 319:640-646, Lee, et al. (1986) Proc. Natl. Acad. Sci. USA 83:2061-2063, and accession code Swissprot 05112 (the accession code for murine

IL-4 is Swissprot 07750) , which are each incorporated herein by reference.

The nucleotide and amino acid sequences of human IL-5 are well known and set forth in Campbell, et al. (1987) Proc. Natl. Acad. Sci. USA 84:6629-6633, Tanabe, et al. (1987) J. Biol. Chem. 262:16580-16584, Campbell, et al. (1988) Eur. J. Biochem. 174:345-352, Azuma, et al. (1986) Nucl. Acids Res. 14:9149-9158, Yokota, et al. (1986) Proc. Natl. Acad. Sci. USA 84:7388-7392, and accession code Swissprot PO5113, which are each incorporated herein by reference.

The nucleotide and amino acid sequences of human IL-10 are well known and set forth in Viera, et al. (1991) Proc. Natl. Acad. Sci. USA 88:1172-1176, and accession code Swissprot P22301.

The nucleotide and amino acid sequences of human IL-15 are well known and set forth in Grabstein, et al. (1994) Science 264:965-968, and accession code Swissprot U03099, which are each incorporated herein by reference.

The nucleotide and amino acid sequences of human IL-18 are well known and set forth in Ushio, et al. (1996) J. Immunol. 156:4274-4279, and accession code D49950, which are each incorporated herein by reference.

The nucleotide and amino acid sequences of human TNF-α are well known and set forth in Pennica, (1984) Nature 312:724-729, and accession code Swissprot PO1375, which are each incorporated herein by reference.

The nucleotide and amino acid sequences of human TNF-β are well known and set forth in Gray, (1984) Nature 312:721-724, and accession code Swissprot PO1374, which are each incorporated herein by reference.

DNA vaccines are described in U.S. Patent No. 5,593,972, U.S. Patent No. 5,589,466, PCT/US90/01515, PCT/US93/02338, PCT/US93/048131, and PCT/US94/00899, and the priority applications cited therein each of the patents and published patent applications, which are each incorporated herein by reference. In addition to the delivery protocols described in those applications, alternative methods of

delivering DNA are described in U.S. Patent Nos. 4,945,050 and 5,036,006, which are both incorporated herein by reference.

An improvement of the present invention relates to the inclusion of genetic material for the co-production of an immunomodulating protein in addition to the production of the antigenic target encoded by nucleic acid sequences of the DNA vaccines.

The present invention relates to methods of introducing genetic material into the cells of an individual in order to induce immune responses against proteins and peptides which are encoded by the genetic material. The methods comprise the steps of administering to the tissue of said individual, either a single nucleic acid molecule that comprises a nucleotide sequence that encodes a target protein and a nucleotide sequence that encodes an immunomodulating protein, or a composition having two nucleic acid molecules, one that comprises a nucleotide sequence that encodes a target protein and one that comprises a nucleotide sequence that encodes an immunomodulating protein. The nucleic acid molecule(s) may be provided as plasmid DNA, the nucleic acid molecules of recombinant vectors or as part of the genetic material provided in an attenuated vaccine.

According to the present invention, compositions and methods are provided which prophylactically and/or therapeutically immunize an individual against a pathogen or abnormal, disease-related cell. The genetic material that encodes a target protein, i.e. a peptide or protein that shares at least an epitope with an immunogenic protein found on the pathogen or cells to be targeted, and genetic material that encodes an immunomodulating protein. The genetic material is expressed by the individual's cells and serves as an immunogenic target against which an immune response is elicited. The resulting immune response reacts with a pathogen or cells to be targeted and is broad based: in addition to a humoral immune response, both arms of the cellular immune response are elicited. The methods of the present invention are useful for conferring prophylactic and

therapeutic immunity. Thus, a method of immunizing includes both methods of protecting an individual from pathogen challenge, or occurrence or proliferation of specific cells as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease.

As used herein the term "target protein" is meant to refer to peptides and protein encoded by gene constructs of the present invention which act as target proteins for an immune response. The term "target protein" refers to a protein against which an immune response can be elicited. The target protein is an immunogenic protein which shares at least an epitope with a protein from the pathogen or undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease against which immunization is required. The immune response directed against the target protein will protect the individual against and treat the individual for the specific infection or disease with which the target protein is associated. The target protein does not need to be identical to the protein against which an immune response is desired. Rather, the target protein must be capable of inducing an immune response that cross reacts to the protein against which the immune response is desired.

The present invention is useful to elicit broad immune responses against a target protein, i.e. proteins specifically associated with pathogens or the individual's own "abnormal" cells. The present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

According to the present invention, DNA or RNA that encodes a target protein and an immunomodulating protein is introduced into the cells of tissue of an individual where it is expressed, thus producing the target protein. The DNA or RNA sequences encoding the target protein and an immunomodulating protein are linked to regulatory elements necessary for expression in the cells of the individual. Regulatory elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as a Kozak region, may also be included in the genetic construct.

As used herein, the term "expressible form" refers to gene constructs which contain the necessary regulatory elements operable linked to a coding sequence that encodes a target protein or immunomodulating protein, such that when present in the cell of the individual, the coding sequence will be expressed.

As used herein, the term "sharing an epitope" refers to proteins which comprise at least one epitope that is identical to or substantially similar to an epitope of another protein.

As used herein, the term "substantially similar epitope" is meant to refer to an epitope that has a structure which is not identical to an epitope of a protein but nonetheless invokes an cellular or humoral immune response which cross reacts to that protein.

Genetic constructs comprise a nucleotide sequence that encodes a target protein and/or immunomodulating protein operably linked to regulatory elements needed for gene expression. According to the invention, combinations of gene constructs which include one that comprises an expressible form of the nucleotide sequence that encodes a target protein and one that includes an expressible form of the nucleotide sequence that encodes immunomodulating protein are provided. Incorporation into a living cell of the DNA or RNA molecule(s) which include the combination of gene constructs results in the expression of the DNA or RNA and production of the target

protein and immunomodulating protein. An enhanced immune response against the target protein results.

When taken up by a cell, the genetic construct(s) may remain present in the cell as a functioning extrachromosomal molecule and/or integrate into the cell's chromosomal DNA. DNA may be introduced into cells where it remains as separate genetic material in the form of a plasmid or plasmids. Alternatively, linear DNA which can integrate into the chromosome may be introduced into the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication. Gene constructs may remain part of the genetic material in attenuated live microorganisms or recombinant microbial vectors which live in cells. Gene constructs may be part of genomes of recombinant viral vaccines where the genetic material either integrates into the chromosome of the cell or remains extrachromosomal.

Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression of the sequence that encodes the target protein or the immunomodulating protein. It is necessary that these elements be operable linked to the sequence that encodes the desired proteins and that the regulatory elements are operably in the individual to whom they are administered.

Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metalothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal which is in pCEP4 plasmid (Invitrogen, San Diego CA), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

In some preferred embodiments related to immunization applications, nucleic acid molecule(s) are

delivered which include nucleotide sequences that encode a target protein, IL-12 protein and, additionally, genes for proteins which further enhance the immune response against such target proteins. Examples of such genes are those which encode cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), GC-SF, GM-CSF, TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and B7.2. In some embodiments, it is preferred that the gene for GM-CSF is included in genetic constructs used in immunizing compositions.

An additional element may be added which serves as a target for cell destruction if it is desirable to eliminate cells receiving the genetic construct for any reason. A herpes thymidine kinase (tk) gene in an expressible form can be included in the genetic construct. The drug gangcyclovir can be administered to the individual and that drug will cause the selective killing of any cell producing tk, thus, providing the means for the selective destruction of cells with the genetic construct.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce DNA constructs which are functional in the cells.

Examples two types of backbones include one type for use in two plasmid systems, and one type for use in single plasmid systems. In two plasmid systems, one plasmid has an expressible form of target coding sequence and one has an expressible form of IL-12 coding sequence. In single plasmid systems, the single plasmid contains expressible forms of both target coding sequence and IL-12 coding sequence.

The method of the present invention comprises the steps of administering nucleic acid molecules to tissue of the individual. In some preferred embodiments, the nucleic acid molecules are administered intramuscularly, intranasally, intraperatoneally, subcutaneously, intradermally,

intravenously, by aerosol administration to lung tissue or topically or by lavage to mucosal tissue selected from the group consisting of vaginal, rectal, urethral, buccal and sublingual.

In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with administration of a facilitating agent. Facilitating agents are also referred to as polynucleotide function enhancers or genetic vaccine facilitator agents. Facilitating agents are described in U.S. Serial Number 08/008,342 filed January 26, 1993, U.S. Serial Number 08/029,336 filed March 11, 1993, U.S. patent Number 5,593,972 issued January 14, 1997 and International Application Serial Number PCT/US94/00899 filed January 26, 1994, which are each incorporated herein by reference. In addition, facilitating agents are described in PCT/US95/12502 filed 9/28/95 and PCT/US95/04071 filed 3/30/95, which are each incorporated herein by reference. Facilitating agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules. In addition, other agents which may function transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with or without a facilitating agent include growth factors, cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), GC-SF, GM-CSF, TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 and B7.2 as well as fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicles such as squalene and squalene, and hyaluronic acid.

In some preferred embodiments, the genetic constructs of the invention are formulated with or administered in conjunction with a facilitator selected from the group consisting of benzoic acid esters, anilides,

amidines, urethans and the hydrochloride salts thereof such as those of the family of local anesthetics.

The facilitators in some preferred embodiments may be a compound having one of the following formulae:

$$Ar - R^1 - O - R^2 - R^3$$

or

$$Ar - N - R^1 - R^2 - R^3$$

or

$$R^4 - N - R^5 - R^6$$

or

$$R^4 - O - R^1 - N - R^7$$

wherein:

Ar is benzene, *p*-aminobenzene, *m*-aminobenzene, *o*-aminobenzene, substituted benzene, substituted *p*-aminobenzene, substituted *m*-aminobenzene, substituted *o*-aminobenzene, wherein the amino group in the aminobenzene compounds can be amino, C_1-C_5 alkylamine, C_1-C_5, C_1-C_5 dialkylamine and substitutions in substituted compounds are halogen, C_1-C_5 alkyl and C_1-C_5 alkoxy;

R^1 is C=O;

R^2 is C_1-C_{10} alkyl including branched alkyls;

R^3 is hydrogen, amine, C_1-C_5 alkylamine, C_1-C_5, C_1-C_5 dialkylamine;

R^2 + R^3 can form a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle;

R^4 is Ar, R^2 or C_1-C_5 alkoxy, a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle and a C_1-C_{10} alkoxy substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle;

R^5 is C=NH;

R^6 is Ar, R^2 or C_1-C_5 alkoxy, a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle and a C_1-C_{10} alkoxy substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle; and.

R^7 is Ar, R^2 or C_1-C_5 alkoxy, a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle and a C_1-C_{10} alkoxy substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle.

Examples of esters include: benzoic acid esters such as piperocaine, meprylcaine and isobucaine; *para*-aminobenzoic acid esters such as procaine, tetracaine, butethamine, propoxycaine and chloroprocaine; *meta*-aminobenzoic acid esters including metabuthamine and primacaine; and *para*-ethoxybenzoic acid esters such as parethoxycaine. Examples of anilides include lidocaine, etidocaine, mepivacaine, bupivacaine, pyrrocaine and prilocaine. Other examples of such compounds include dibucaine, benzocaine, dyclonine, pramoxine, proparacaine, butacaine, benoxinate, carbocaine, methyl bupivacaine, butasin picrate, phenacaine, diothan, luccaine, intracaine, nupercaine, metabutoxycaine, piridocaine, biphenamine and the botanically-derived bicyclics such as cocaine, cinnamoylcocaine, truxilline and cocaethylene and all such compounds complexed with hydrochloride.

In preferred embodiments, the facilitator is bupivacaine. The difference between bupivacaine and mepivacaine is that bupivacaine has a N-butyl group in place of an N-methyl group of mepivacaine. Compounds may have at that N, C_1-C_{10}. Compounds may be substituted by halogen such as procaine and chloroprocaine. The anilides are preferred.

The facilitating agent is administered prior to, simultaneously with or subsequent to the genetic construct. The facilitating agent and the genetic construct may be formulated in the same composition.

Bupivacaine-HCl is chemically designated as 2-piperidinecarboxamide, 1-butyl-N-(2,6-dimethylphenyl)-monohydrochloride, monohydrate and is widely available commercially for pharmaceutical uses from many sources including from Astra Pharmaceutical Products Inc. (Westboro, MA) and Sanofi Winthrop Pharmaceuticals (New York, NY), Eastman Kodak (Rochester, NY). Bupivacaine is commercially formulated with and without methylparaben and with or without epinephrine. Any such formulation may be used. It is commercially available for pharmaceutical use in concentration of 0.25%, 0.5% and 0.75% which may be used on the invention. Alternative concentrations, particularly those between 0.05% - 1.0% which elicit desirable effects may be prepared if desired. According to the present invention, about 250 g to about 10 mg of bupivacaine is administered. In some embodiments, about 250 g to about 7.5 mg is administered. In some embodiments, about 0.05 mg to about 5.0 mg is administered. In some embodiments, about 0.5 mg to about 3.0 mg is administered. In some embodiments about 5 to 50 g is administered. For example, in some embodiments about 50 μl to about 2 ml, preferably 50 μl to about 1500 μl and more preferably about 1 ml of 0.25-0.50% bupivacaine-HCl and 0.1% methylparaben in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with or after the vaccine is administered. Similarly, in some embodiments, about 50 μl to about 2 ml, preferably 50 μl to about 1500 μl and more preferably about 1 ml of 0.25-0.50% bupivacaine-HCl in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with or after the vaccine is administered. Bupivacaine and any other similarly acting compounds, particularly those of the related family of local anesthetics may be administered at concentrations which provide the desired facilitation of uptake of genetic constructs by cells.

In some embodiments of the invention, the individual is first subject to injection of the facilitator prior to

adminstration of the genetic construct. That is, up to, for example, up to a about a week to ten days prior to administration of the genetic construct, the individual is first injected with the facilitator. In some embodiments, the individual is injected with facilitator about 1 to 5 days, in some embodiments 24 hours, before or after administration of the genetic construct. Alternatively, if used at all, the facilitator is administered simultaneously, minutes before or after adminstration of the genetic construct. Accordingly, the facilitator and the genetic construct may be combined to form a single pharmaceutical compositions.

In some embodiments, the genetic constructs are administered free of facilitating agents, that is in formulations free from facilitating agents using adminstration protocols in which the genetic constructions are not administered in conjunction with the administration of facilitating agents.

Nucleic acid molecules which are delivered to cells according to the invention may serve as genetic templates for proteins that function as prophylactic and/or therapeutic immunizing agents. In preferred embodiments, the nucleic acid molecules comprise the necessary regulatory sequences for transcription and translation of the coding region in the cells of the animal.

The present invention may be used to immunize an individual against all pathogens such as viruses, prokaryote and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize an individual against those pathogens which infect cells and which are not encapsulated such as viruses, and prokaryote such as gonorrhea, listeria and shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens which include a stage in the life cycle where they are intracellular pathogens. As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, at least part of its reproductive

or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins. Table 1 provides a listing of some of the viral families and genera for which vaccines according to the present invention can be made. DNA constructs that comprise DNA sequences which encode the peptides that comprise at least an epitope identical or substantially similar to an epitope displayed on a pathogen antigen such as those antigens listed on the tables are useful in vaccines. Moreover, the present invention is also useful to immunize an individual against other pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites such as those listed on Table 2.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material which encodes immunogenic proteins against which a protective immune response can be mounted must be included in a genetic construct as the coding sequence for the target. Whether the pathogen infects intracellularly, for which the present invention is particularly useful, or extracellularly, it is unlikely that all pathogen antigens will elicit a protective response. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The genetic construct used in the genetic vaccine can include genetic material which encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets.

Tables 1 and 2 include lists of some of the pathogenic agents and organisms for which genetic vaccines can be prepared to protect an individual from infection by them. In some preferred embodiments, the methods of immunizing an individual against a pathogen are directed against HIV, HTLV or HBV.

Another aspect of the present invention provides a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic in

hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

It has been discovered that introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell"-associated protein into the cells of an individual results in the production of those proteins in the vaccinated cells of an individual. As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. To immunize against hyperproliferative diseases, a genetic construct that includes a nucleotide sequence which encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which are proteins encoded by oncogenes such as *myb*, *myc*, *fyn*, and the translocation gene *bcr/abl*, *ras*, *src*, P53, *neu*, *trk* and EGRF. In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are

also used target antigens for autoimmune disease. Other tumor-associated proteins can be used as target proteins such as proteins which are found at higher levels in tumor cells including the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and technology as well as epidemiology allow for the determination of probability and risk assessment for the development of cancer in individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of genetic constructs serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against

targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include Vβ-3, Vβ-14, Vβ-17 and Vα-17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell, M.D., *et al.*, 1991 *Proc. Natl. Acad. Sci. USA* 88:10921-10925; Paliard, X., *et al.*, 1991 *Science* 253:325-329; Williams, W.V., *et al.*, 1992 *J. Clin. Invest.* 90:326-333; each of which is incorporated herein by reference.

In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-7 and Vα-10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K.W., *et al.*, 1990 *Science* 248:1016-1019; Oksenberg, J.R., *et al.*, 1990 *Nature* 345:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-6, Vβ-8, Vβ-14 and Vα-16, Vα-3C, Vα-7, Vα-14, Vα-15, Vα-16, Vα-28 and Vα-12. Thus, vaccination with a DNA construct that encodes at least one of

these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, *et al.* 1987 *Sequence of Proteins of Immunological*

Interest U.S. Department of Health and Human Services, Bethesda MD, which is incorporated herein by reference. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* 87:1066, which is incorporated herein by reference.

In addition to using expressible forms of immunomodulating protein coding sequences to improve genetic vaccines, the present invention relates to improved attenuated live vaccines and improved vaccines which use recombinant vectors to deliver foreign genes that encode antigens. Examples of attenuated live vaccines and those using recombinant vectors to deliver foreign antigens are described in U.S. Patent Nos.: 4,722,848; 5,017,487; 5,077,044; 5,110,587; 5,112,749; 5,174,993; 5,223,424; 5,225,336; 5,240,703; 5,242,829; 5,294,441; 5,294,548; 5,310,668; 5,387,744; 5,389,368; 5,424,065; 5,451,499; 5,453,364; 5,462,734; 5,470,734; and 5,482,713, which are each incorporated herein by reference. Gene constructs are provided which include the nucleotide sequence that encodes an immunomodulating protein is operably linked to regulatory sequences that can function in the vaccinee to effect expression. The gene constructs are incorporated in the attenuated live vaccines and recombinant vaccines to produce improved vaccines according to the invention.

The present invention provides an improved method of immunizing individuals that comprises the step of delivering gene constructs to the cells of individuals as part of vaccine compositions which include are provided which include DNA vaccines, attenuated live vaccines and recombinant vaccines. The gene constructs comprise a nucleotide sequence that encodes an immunomodulating protein and that is operably linked to regulatory sequences that can function in the vaccinee to effect expression. The improved vaccines result in an enhanced cellular immune response.

In some aspects of the invention, the nucleic acid molecules encoding a human immunomodulating protein are

delivered as a gene therapeutic i.e. without co-administration of an immunogenic target or nucleic acid molecule encoding an immunogenic target protein. The gene therapy aspects arise from function of the immunomodulator proteins which drives the immune responses.

Genetic constructs that are delivered as gene therapeutics are nucleic acid molecules that encode human IL-12. Such genetic constructs are preferably plasmids. Also contemplated are other nucleic acid based vectors such as recombinant viruses, recombinant microorganisms and linear nucleic acid molecules, all of which are well known to those having ordinary skill in the art. Plasmids useful for adminstration to individuals in whose tissue the plasmid is taken up and expressed are well known. Contemplated recombinant viruses include: recombinant vaccinia virus vectors, recombinant adenovirus virus vectors, and recombinant retroviral vectors. Contemplated recombinant organisms include: recombinant BCG vectors, and recombinant *Salmonella* vectors. Linear nucleic acid molecules as well as plasmid DNA molecules may be encapsulated in microspheres and liposomes.

In some preferred embodiments, plasmids that encode human an immunomodulating protein are those genetic constructs described above. Essentially, the same compositions and methods may be used for gene therapeutics that encode human immunomodulating proteins as described for genetic immunization compositions and methods that include gene constructs which encode human immunomodulating protein except the gene therapy compositions do not include coding sequences for immune target proteins. This disclosure is intended to describe gene therapeutics that encode human immunomodulating proteins by referring to the above gene constructs. The gene constructs described above which include nucleotide sequences that encode human immunomodulating protein operably linked to regulatory elements necessary for expression in an individual are intended to describe gene therapeutics. Such constructs may be administered to individuals in order to modify and/or drive an immune response.

For example, IL-12 may be delivered to treat an individual suffering from an allergy, cancer, autoimmune diseases or infections. Genetic constructs that encode IL-12 are administered with or without facilitators. In some embodiments, the genetic constructs are administered in conjunction with one or more of the facilitating agents described above. In some embodiments, the gene construct is delivered free of any facilitating agent. In some preferred embodiments, the gene encoding IL-12 is free of any infectious agents. In some embodiments, the gene construct is administered using a needleless injection device. In some embodiments, the gene encoding IL-12 is delivered using microprojectiles. In some embodiments, the gene encoding IL-12 is delivered free of any solid particles.

The pharmaceutical compositions according to the present invention which are either genetic vaccines or gene therapy compositions comprise about 1 nanogram to about 1000 micrograms of DNA. In some preferred embodiments, the compositions contain about 10 nanograms to about 800 micrograms of DNA. In some preferred embodiments, the compositions contain about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the compositions contain about 1 to about 350 micrograms of DNA. In some preferred embodiments, the compositions contain about 25 to about 250 micrograms of DNA. In some preferred embodiments, the compositions contain about 100 micrograms DNA.

The pharmaceutical compositions according to the present invention are formulated according to the mode of administration to be used. One having ordinary skill in the art can readily formulate a pharmaceutical composition that comprises a genetic construct. In cases where intramuscular injection is the chosen mode of administration, an isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a

vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free.

The methods of the present invention are useful in the fields of both human and veterinary medicine. Accordingly, the present invention relates to genetic immunization of mammals, birds and fish. The methods of the present invention can be particularly useful for mammalian species including human, bovine, ovine, porcine, equine, canine and feline species.

A novel immunomodulator, human BL1, has been discovered. The DNA and predicted amino acid sequences are set forth in Figure 14. This protein and fragments thereof enhance immune responses when co-administered with vaccine compositions that can introduce an immunogen to the individual. As used herein, the term "immunomodulating fragment" is meant to refer to a fragment of BL1 that is less than the full length sequence shown in Figure 14 but which retains the immunodulating activity of the full length compound. According to the invention, delivery of the BL1 gene sequence results in immunomodulation. In some experiments, protein expression was not detected but imnmune responses were nonetheless affected by co-delivery indicating that the delivery of the BL1 DNA is the critical step to modulating and directing immune responses. As set forth herein, the disclosure is intended to disclose the use of BL1 DNA in immunodulating compositions and methods irrespectiuve of protein production. Accordingly, the disclosure is intended to relate to methods of using BL1 DNA and vecotrs comprising the same as immunomodulating agents and in immunomodulaing compositions useful to manipulate and direct immune responses either as primary active agents or as co-agents administered in conjunction with immunogens or DNa that encodes immunogens.

Protein encoded by BL1 DNa may be isolated and purified; hybridomas which produce antibodies that bind to the protein can be generated; cDNAs that encode this protein have

been isolated, sequenced, incorporated into vectors including expression vector which were introduced into host cells that then express the proteins recombinantly. Co-administration of the vectors with vectors that encode immunogens resulted in enhanced immune responses against the immunogen.

The discovery of BL1 provides the means to design and utilize vaccination protocols which enhance, drive and direct immune responses.

Isolated cDNA that encodes BL1 is useful as a starting material in the construction of recombinant expression vectors that can produce BL1 or immunomodulating fragments thereof. The cDNA is incorporated into vectors including expression vectors which are introduced into host cells that then express the proteins recombinantly. Nucleic acid molecules and fragments thereof may be used as probes to detect the presence of the BL1 coding sequence. Such probes hybridize specifically to BL1 coding sequences. As used herein, the term "specific BL1 sequence" is meant to refer to those sequences which are unique to BL1. Nucleic acid molecules which comprise a nucleotide sequence which are complementary to specific fragments of the cDNA that encode BL1 may be used as antisense molecules and primers to inhibit translation of mRNA and amplify genetic sequences, respectively.

BL1 is encoded by cDNA shown in Figure 14 and has a predicted amino acid sequence shown in Figure 14. BL1 coding sequences can be synthesized routinely and BL1 protein can be produced by recombinant DNA methods or synthesized by standard protein synthesis techniques.

Using standard techniques and readily available starting materials, a nucleic acid molecule that encodes BL1 may be prepared. The present invention relates to an isolated nucleic acid molecule that comprises a nucleotide sequence that encodes BL1. The present invention relates to an isolated nucleic acid molecule that comprises a nucleotide sequence that encodes an amino acid sequence of Figure 14, or an immunomodulating fragment thereof. In some embodiments,

the nucleic acid molecules consist of a nucleotide sequence that encodes BL1. In some embodiments, the nucleic acid molecules comprise the nucleotide sequence that consists of the coding sequence in Figure 14. In some embodiments, the nucleic acid molecules consist of the nucleotide sequence set forth in Figure 14. The isolated nucleic acid molecules of the invention are useful to prepare constructs and recombinant expression vectors.

The probes or primers that are specific for BL1 have at least 16 nucleotides, preferably at least 24 nucleotides. The probes or primers are used to screen the cDNA library using standard hybridization techniques.

The cDNA that encodes BL1 may be used to design PCR primers for amplifying nucleic acid sequences. PCR technology is practiced routinely by those having ordinary skill in the art and its uses in diagnostics are well known and accepted. Methods for practicing PCR technology are disclosed in "PCR Protocols: A Guide to Methods and Applications", Innis, M.A., *et al.* Eds. Academic Press, Inc. San Diego, CA (1990) which is incorporated herein by reference. Applications of PCR technology are disclosed in "Polymerase Chain Reaction" Erlich, H.A., *et al.*, Eds. Cold Spring Harbor Press, Cold Spring Harbor, NY (1989) which is incorporated herein by reference. Some simple rules aid in the design of efficient primers. Typical primers are 18-28 nucleotides in length having 50% to 60% g+c composition. The entire primer is preferably complementary to the sequence it must hybridize to. Preferably, primers generate PCR products 100 base pairs to 2000 base pairs. However, it is possible to generate products of 50 base pairs to up to 10 kb and more.

PCR technology allows for the rapid generation of multiple copies of nucleotide sequences by providing 5' and 3' primers that hybridize to sequences present in a nucleic acid molecule, and further providing free nucleotides and an enzyme which fills in the complementary bases to the nucleotide sequence between the primers with the free nucleotides to produce a complementary strand of DNA. The

enzyme will fill in the complementary sequences adjacent to the primers. If both the 5' primer and 3' primer hybridize to nucleotide sequences on the complementary strands of the same fragment of nucleic acid, exponential amplification of a specific double-stranded product results. If only a single primer hybridizes to the nucleic acid molecule, linear amplification produces single-stranded products of variable length.

The present invention relates to a vector or a recombinant expression vector that comprises a nucleotide sequence that encodes BL1 that comprises an amino acid sequence in Figure 14. As used herein, the term "recombinant expression vector" is meant to refer to a plasmid, phage, viral particle or other vector which, when introduced into an appropriate host, contains the necessary genetic elements to direct expression of the coding sequence that encodes BL1. One having ordinary skill in the art can insert a nucleic acid molecule that encodes BL1 into an expression vector using standard techniques and readily available starting materials. The coding sequence is operably linked to the necessary regulatory sequences. Expression vectors are well known and readily available. Examples of expression vectors include plasmids, phages, viral vectors and other nucleic acid molecules or nucleic acid molecule containing vehicles useful to transform host cells and facilitate expression of coding sequences. In some embodiments, the recombinant expression vector comprises the nucleotide sequence set forth in Figure 14. The recombinant expression vectors of the invention are preferably plasmids.

The present invention relates to a host cell that comprises the recombinant expression vector that includes a nucleotide sequence that encodes BL1. In some embodiments, the host cell comprises a recombinant expression vector that comprises the nucleotide sequence in Figure 14. Host cells for use in well known recombinant expression systems for production of proteins are well known and readily available. Examples of host cells include bacteria cells such as *E. coli*,

yeast cells such as *S. cerevisiae*, insect cells such as *S. frugiperda*, non-human mammalian tissue culture cells chinese hamster ovary (CHO) cells and human tissue culture cells such as HeLa cells.

In some embodiments, for example, one having ordinary skill in the art can, using well known techniques, insert DNA molecules into a commercially available expression vector for use in well known expression systems. For example, the commercially available plasmid pSE420 (Invitrogen, San Diego, CA) may be used for production of BL1 in *E. coli*. The commercially available plasmid pYES2 (Invitrogen, San Diego, CA) may, for example, be used for production in *S. cerevisiae* strains of yeast. The commercially available MAXBAC™ complete baculovirus expression system (Invitrogen, San Diego, CA) may, for example, be used for production in insect cells. The commercially available plasmid pcDNA I or pcDNA3 (Invitrogen, San Diego, CA) may, for example, be used for production in mammalian cells such as Chinese Hamster Ovary cells. One having ordinary skill in the art can use these commercial expression vectors and systems or others to produce hVIP routine techniques and readily available starting materials. (*See e.g.*, Sambrook et al., *Molecular Cloning a Laboratory Manual*, Second Ed. Cold Spring Harbor Press (1989) which is incorporated herein by reference.) Thus, the desired proteins can be prepared in both prokaryotic and eukaryotic systems, resulting in a spectrum of processed forms of the protein.

One having ordinary skill in the art may use other commercially available expression vectors and systems or produce vectors using well known methods and readily available starting materials. Expression systems containing the requisite control sequences, such as promoters and polyadenylation signals, and preferably enhancers, are readily available and known in the art for a variety of hosts. *See e.g.*, Sambrook et al., *Molecular Cloning a Laboratory Manual*, Second Ed. Cold Spring Harbor Press (1989).

The expression vector including the DNA that encodes BL1 is used to transform the compatible host which is then

cultured and maintained under conditions wherein expression of the foreign DNA takes place. The protein of the present invention thus produced is recovered from the culture, either by lysing the cells or from the culture medium as appropriate and known to those in the art. One having ordinary skill in the art can, using well known techniques, isolate BL1 that is produced using such expression systems. The methods of purifying BL1 from natural sources using antibodies which specifically bind to BL1 as described above, may be equally applied to purifying BL1 produced by recombinant DNA methodology.

Examples of genetic constructs include the BL1 coding sequence operably linked to a promoter that is functional in a human as set forth above or a cell line into which the constructs are transfected. Examples of constitutive promoters include promoters from cytomegalovirus or SV40. Examples of inducible promoters include mouse mammary leukemia virus or metallothionein promoters.

In addition to producing BL1 by recombinant techniques, automated peptide synthesizers may also be employed to produce BL1. Such techniques are well known to those having ordinary skill in the art and are useful if derivatives which have substitutions not provided for in DNA-encoded protein production.

Nucleic acid molecules that encode BL1 may be delivered using any one of a variety of delivery components, such as direct plasmid administration, recombinant viral expression vectors or other suitable delivery means, so as to affect their introduction and expression in an individual or compatible host cells. In general, viral vectors may be DNA viruses such as recombinant adenoviruses and recombinant vaccinia viruses or RNA viruses such as recombinant retroviruses. Other recombinant vectors include recombinant prokaryotes which can infect cells and express recombinant genes. In addition to recombinant vectors, other delivery components are also contemplated such as encapsulation in liposomes, transferrin-mediated transfection and other

receptor-mediated means. The invention is intended to include such other forms of expression vectors and other suitable delivery means which serve equivalent functions and which become known in the art subsequently hereto.

The present invention also relates to a transgenic non-human mammal that comprises the recombinant expression vector that comprises a nucleic acid sequence that encodes BL1. Transgenic non-human mammals useful to produce recombinant proteins are well known as are the expression vectors necessary and the techniques for generating transgenic animals. Generally, the transgenic animal comprises a recombinant expression vector in which the nucleotide sequence that encodes BL1 is operably linked to a mammary cell specific promoter whereby the coding sequence is only expressed in mammary cells and the recombinant protein so expressed is recovered from the animal's milk. One having ordinary skill in the art using standard techniques, such as those taught in U.S. Patent No. 4,873,191 issued October 10, 1989 to Wagner and U.S. Patent No. 4,736,866 issued April 12, 1988 to Leder, both of which are incorporated herein by reference, can produce transgenic animals which produce BL1. Preferred animals are goats, sheep or rodents, particularly rats and mice.

BL1 protein or expression vectors for producing the same can be formulated into pharmaceutical compositions.

Pharmaceutical compositions according to the invention include delivery components in combination with nucleic acid molecules which further comprise a pharmaceutically acceptable carriers or vehicles, such as, for example, saline. Any medium may be used which allows for successful delivery of the nucleic acid. One skilled in the art would readily comprehend the multitude of pharmaceutically acceptable media that may be used in the present invention. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, A. Osol, a standard reference text in this field, which is incorporated herein by reference. The pharmaceutical compositions of the present invention may be

administered by any means that enables the active agent to reach the targeted cells. Because peptides are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, transdermal, intramuscular, would ordinarily be used to optimize absorption. Intravenous administration may be accomplished with the aid of an infusion pump. The pharmaceutical compositions of the present invention may be formulated as an emulsion. Alternatively, they may be formulated as aerosol medicaments for intranasal or inhalation administration. In some cases, topical administration may be desirable.

The dosage administered varies depending upon factors such as: pharmacodynamic characteristics; its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment; and frequency of treatment. Usually, the dosage of peptide can be about 1 to 3000 milligrams per 50 kilograms of body weight; preferably 10 to 1000 milligrams per 50 kilograms of body weight; more preferably 25 to 800 milligrams per 50 kilograms of body weight. Ordinarily 8 to 800 milligrams are administered to an individual per day in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results. Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Compositions for parenteral, intravenous, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives and are preferably sterile and pyrogen free. Pharmaceutical

compositions which are suitable for intravenous administration according to the invention are sterile and pyrogen free.

For parenteral administration, the peptides of the invention can be, for example, formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The vehicle or lyophilized powder may contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques. For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

The pharmaceutical compositions of the present invention may be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intrathecal or intraventricular administration.

Hybridomas which produce antibodies that bind to BL1, and the antibodies themselves, are useful in the isolation and purification of BL1 and protein complexes that include BL1. In addition, antibodies are specific inhibitors of BL1 activity. Antibodies which specifically bind to hVIP may be used to purify the protein from natural sources using well known techniques and readily available starting materials. Such antibodies may also be used to purify the

protein from material present when producing the protein by recombinant DNA methodology.

As used herein, the term "antibody" is meant to refer to complete, intact antibodies, and Fab fragments and $F(ab)_2$ fragments thereof. Complete, intact antibodies include monoclonal antibodies such as murine monoclonal antibodies, chimeric antibodies and humanized antibodies. Antibodies that bind to an epitope is useful to isolate and purify that protein from both natural sources or recombinant expression systems using well known techniques such as affinity chromatography, i.e. the antibodies do not cross react with other proteins. Such antibodies are useful to detect the presence of such protein in a sample and to determine if cells are expressing the protein.

The production of antibodies and the protein structures of complete, intact antibodies, Fab fragments and $F(ab)_2$ fragments and the organization of the genetic sequences that encode such molecules are well known and are described, for example, in Harlow, E. and D. Lane (1988) *ANTIBODIES: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. which is incorporated herein by reference. Briefly, for example, BL1, or an immunogenic fragment thereof, is injected into mice. The spleen of the mouse is removed, the spleen cells are isolated and fused with immortalized mouse cells. The hybrid cells, or hybridomas, are cultured and those cells which secrete antibodies are selected. The antibodies are analyzed and, if found to specifically bind to BL1, the hybridoma which produces them is cultured to produce a continuous supply of antibodies.

The Examples set out below include representative examples of aspects of the present invention. The Examples are not meant to limit the scope of the invention but rather serve exemplary purposes. In addition, various aspects of the invention can be summarized by the following description. However, this description is not meant to limit the scope of the invention but rather to highlight various aspects of the invention. One having ordinary skill in the art can readily

appreciate additional aspects and embodiments of the invention.

EXAMPLES

Example 1

The following is a list of constructs which may be used in the methods of the present invention. The vector pBabe.puro, which is used as a starting material to produce many of the below listed constructs, was originally constructed and reported by Morgenstern, J.P. and H. Land, 1990 *Nucl. Acids Res.* 18(12):3587-3596, which is incorporated herein by reference. The pBabe.puro plasmid is particularly useful for expression of exogenous genes in mammalian cells. DNA sequences to be expressed are inserted at cloning sites under the control of the Moloney murine leukemia virus (Mo MuLV) long terminal repeat (LTR) promoter. The plasmid contains the selectable marker for puromycin resistance.

Plasmid pBa.Vα3-IL-12 is a plasmid that contains a 2.7 kb *Eco*RI genomic fragment encoding the T cell receptor Va3 region containing the L, V and J segments cloned into the *Eco*RI site of pBabe.puro and the IL-12 coding sequence operably linked to the CMV promoter and SV40 polyadenylation sequence. The T cell receptor-derived target protein is useful in the immunization against and treatment of T cell mediated autoimmune disease and clonotypic T cell lymphoma and leukemia.

Plasmid pBa.*gagpol-vpr*-IL-12 is a plasmid that contains the *gag/pol* and *vif* genes from HIV/MN cloned into pBabe.puro. The *vpr* gene is deleted. The plasmid which contains these HIV viral genes, which encode HIV target proteins, and the IL-12 coding sequence operably linked to the CMV promoter and SV40 polyadenylation sequence is useful in the immunization against and treatment of HIV infection and AIDS. The HIV DNA sequence is published in Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pM160 is a plasmid that contains the 2.3 kb PCR fragment encoding the HIV-I/3B envelope protein and *rev/tat* genes cloned into pMAMneoBlue. The *nef* region is deleted. The plasmid which contains these HIV viral genes, which encode HIV target proteins, and the IL-12 coding sequence operably linked to the CMV promoter and SV40 polyadenylation sequence is useful in the immunization against and treatment of HIV infection and AIDS. The DNA sequence of HIV-1/3B is published in Fisher, A., 1985 *Nature* 316:262, which is incorporated herein by reference. The sequence is accessible from Genbank No.: K03455, which is incorporated herein by reference.

Plasmid pBa.VL-IL-12 is a plasmid that contains PCR fragment encoding the VL region of an anti-DNA antibody cloned into pBabe.puro at the *Xba*I and *Eco*RI sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The antibody-derived target protein is an example of a target protein useful in the immunization against and treatment of B cell mediated autoimmune disease and clonotypic B cell lymphoma and leukemia. A general method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* 87:1066, which is incorporated herein by reference.

Plasmid pOspA.B-IL-12 is a plasmid which contains the coding regions encoding the OspA and OspB antigens of the *Borrelia burgdorferi*, the spirochete responsible for Lyme's disease cloned into pBabe.puro at the *Bam*HI and *Sal*I sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. See: Williams, W.V., *et al.* 1992 *DNA and Cell. Biol.* 11(3):207, which is incorporated herein by reference. The plasmid which contains these pathogen genes, which encode target proteins, is useful in the immunization against Lyme's disease.

Plasmid pBa.Rb-G-IL-12 is a plasmid which contains a PCR generated fragment encoding the rabies G protein cloned into pBabe.puro at the *Bam*HI site and the IL-12 coding sequence operably linked ot the CMV promoter and SV40

polyadenylation sequence. The plasmid which contains this pathogen gene, which encodes the rabies G protein, is useful in the immunization against Rabies. The DNA sequence is disclosed in Genebank No.:M32751, which is incorporated herein by reference. See also: Anilionis, A., *et al.*, 1981 *Nature* 294:275, which is incorporated herein by reference.

Plasmid pBa.HPV-L1 is a plasmid which contains a PCR generated fragment encoding the L1 capsid protein of the human papillomavirus (HPV) including HPV strains 16, 18, 31 and 33 cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The plasmid is useful in the immunization against HPV infection and the cancer caused thereby. The DNA sequence is disclosed in Genebank No.:M15781, which is incorporated herein by reference. See also: Howley, P., 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al.* Chapter 58:1625; and Shah, K. and P. Howley, 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al.* Chapter 59; both of which are incorporated herein by reference.

Plasmid pBa.HPV-L2-IL-12 is a plasmid which contains a PCR generated fragment encoding the L2 capsid protein of the human papillomavirus (HPV) including HPV strains 16, 18, 31 and 33 cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The plasmid is useful in the immunization against HPV infection and the cancer caused thereby. The DNA sequence is disclosed in Genebank No.:M15781, which is incorporated herein by reference. See also: Howley, P., 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al.* Chapter 58:1625; and Shah, K. and P. Howley, 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al.* Chapter 59; both of which are incorporated herein by reference.

Plasmid pBa.MNp7-IL-12 is a plasmid which contains a PCR generated fragment encoding the p7 coding region including the HIV MN *gag* (core protein) sequence cloned into

pBabe.puro at the *Bam*HI site and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated herein by reference.

Plasmid pGA733-2-IL-12 is a plasmid that contains the GA733-2 tumor surface antigen cloned from the colorectal carcinoma cell line SW948 into pCDM8 vector (Seed, B. and A. Aruffo, 1987 *Proc. Natl. Acad. Sci. USA* 84:3365, which is incorporated herein by reference) at *Bst*XI site and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The tumor-associated target protein is an example of a target protein useful in the immunization against and treatment of hyperproliferative disease such as cancer. The GA733-2 antigen is a useful target antigen against colon cancer. The GA733 antigen is reported in Szala, S. *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* 87:3542-3546, which is incorporated herein by reference.

Plasmid pT4-pMV7-IL-12 is a plasmid that contains cDNA which encodes human CD4 receptor cloned into pMV7 vector at the *Eco*RI site and the IL-12 coding sequence cperably linked ot the CMV promoter and SV40 polyadenylation sequence. The CD4 target protein is useful in the immunization against and treatment of T cell lymphoma. Plasmid pT4-pMV7 is available from the AIDS Repository, Catalog No. 158.

Plasmid pDJGA733-IL-12 is a plasmid that contains the GA733 tumor surface antigen cloned into pBabe.puro at the *Bam*HI site and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The tumor-associated target protein is an example of a target protein useful in the immunization against and treatment of hyperproliferative disease such as cancer. The GA733 antigen is a useful target antigen against colon cancer.

Plasmid pBa.RAS-IL-12 is a plasmid that contains the ras coding region that was first subcloned from pZIPneoRAS and cloned into pBabe.puro at the *Bam*HI site and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The ras target protein is an example of a cytoplasmic signaling molecule. The method of cloning ras is reported in Weinberg 1984 *mol.. Cell. Biol.* 4:1577, which is incorporated herein by reference. Ras encoding plasmid are useful for the immunization against and treatment of hyperproliferative disease such as cancer; in particular, ras related cancer such as bladder, muscle, lung, brain and bone cancer.

Plasmid pBa.MNp55-IL-12 is a plasmid which contains a PCR generated fragment encoding the p55 coding region including the HIV MN gag precursor (core protein) sequence cloned into pBabe.puro at the *Bam*HI site and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated herein by reference.

Plasmid pBa.MNp24-IL-12 is a plasmid which contains a PCR generated fragment from the pMN-SF1 template encoding the p24 coding region including the whole HIV MN gag coding region cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pBa.MNp17-IL-12 is a plasmid which contains a PCR generated fragment encoding the p17 coding region including the HIV MN *gag* (core protein) sequence cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pBa.SIVenv-IL-12 is a plasmid which contains a 2.71 PCR generated fragment amplified from a construct containing SIV 239 in pBR322 cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The plasmid is available from the AIDS Research and Reference Reagent Program; Catalog No. 210.

Plasmid pcTSP/ATK.env-IL-12 is a plasmid which contains a PCR generated fragment encoding the complete HTLV envelope coding region from HTLV-1/TSP and /ATK isolates subcloned into the pcDNA1/neo vector and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. Plasmid pcTSP/ATK.env is reported in 1988 *Proc. Natl. Acad. Sci. USA* 85:3599, which is incorporated herein by reference. The HTLV env target protein is useful in the immunization against and treatment of infection by HTLV and T cell lymphoma.

Plasmid pBa.MNgp160-IL-12 is a plasmid which contains a 2.8 kb PCR generated fragment amplified from a construct containing MNenv in pSP72 and cloned into pBabe.puro at the *BamHI* and *Eco*RI sites and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. Reiz, M.S.; 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated

herein by reference. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS.

Plasmid pC.MNp55-IL-12 is a plasmid which contains a 1.4 kb PCR generated fragment amplified from the *gag* region of MN isolate and cloned into the pCEP4 vector. The plasmid which contains these HIV viral genes, which encode HIV target proteins and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pC.Neu-IL-12 is a plasmid that contains a 3.8 kb DNA fragment containing the human neu oncogene coding region that was cut out from the LTR-2/erbB-2 construct and subcloned into the pCEP4 vector and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The pC.Neu plasmid is reported in DiFiore 1987 *Science* 237:178, which is incorporated herein by reference. The neu oncogene target protein is an example of a growth factor receptor useful as a target protein for the immunization against and treatment of hyperproliferative disease such as cancer; in particular, colon, breast, lung and brain cancer.

Plasmid pC.RAS-IL-12 is a plasmid that contains a 1.4 kb DNA fragment containing the ras oncogene coding region that was first subcloned from pZIPneoRAS and subcloned into pCEP4 at the BamHI site and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence. The pC.RAS plasmid is reported in Weinberg 1984 *Mol.. Cell. Biol.* 4:1577, which is incorporated herein by reference. The ras target protein is an example of a cytoplasmic signalling molecule. Ras encoding plasmid are useful for the immunization against and treatment of hyperproliferative

disease such as cancer; in particular, ras related cancer such as bladder, muscle, lung, brain and bone cancer.

Plasmid pNLpuro-IL-12 is a plasmid which contains HIV *gag/pol* and SV40-puro insertion. The plasmid which contains these HIV viral genes, which encode HIV target proteins and the IL-12 coding sequence operably linked ot the CMV promoter and SV40 polyadenylation sequence, is useful in the immunization against and treatment of HIV infection and AIDS.

Example 2

Plasmid pCSIL-12 contains the IL-12 coding sequences operably linked to CMV promoters and SV40 polyadenylation sequences.

Example 3

Compositions according to some embodiments of the invention may be prepared by combining plasmid pCSIL-12 with any one of the following plasmids.

Plasmid pBa.Vα3 is a 7.8 kb plasmid that contains a 2.7 kb *Eco*RI genomic fragment encoding the T cell receptor Va3 region containing the L, V and J segments cloned into the *Eco*RI site of pBabe.puro. The T cell receptor-derived target protein is useful in the immunization against and treatment of T cell mediated autoimmune disease and clonotypic T cell lymphoma and leukemia.

Plasmid pBa.*gagpol-vpr* is a 9.88 kb plasmid that contains the *gag/pol* and *vif* genes from HIV/MN cloned into pBabe.puro. The *vpr* gene is deleted. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. The HIV DNA sequence is published in Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pM160 is an 11.0 kb plasmid that contains the 2.3 kb PCR fragment encoding the HIV-I/3B envelope protein and *rev/tat* genes cloned into pMAMneoBlue. The *nef* region is

deleted. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. The DNA sequence of HIV-1/3B is published in Fisher, A., 1985 *Nature* 316:262, which is incorporated herein by reference. The sequence is accessible from Genbank No.: K03455, which is incorporated herein by reference.

Plasmid pBa.VL is a 5.4 kb plasmid that contains PCR fragment encoding the VL region of an anti-DNA antibody cloned into pBabe.puro at the *Xba*I and *Eco*RI sites. The antibody-derived target protein is an example of a target protein useful in the immunization against and treatment of B cell mediated autoimmune disease and clonotypic B cell lymphoma and leukemia. A general method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* 87:1066, which is incorporated herein by reference.

Plasmid pOspA.B is a 6.84 kb plasmid which contains the coding regions encoding the OspA and OspB antigens of the *Borrelia burgdorferi*, the spirochete responsible for Lyme's disease cloned into pBabe.puro at the *Bam*HI and *Sal*I sites. Williams, W.V., *et al.* 1992 *DNA and Cell. Biol.* 11(3):207, which is incorporated herein by reference. The plasmid which contains these pathogen genes, which encode target proteins, is useful in the immunization against Lyme's disease.

Plasmid pBa.Rb-G is a 7.10 kb plasmid which contains a PCR generated fragment encoding the rabies G protein cloned into pBabe.puro at the *Bam*HI site. The plasmid which contains this pathogen gene, which encodes the rabies G protein, is useful in the immunization against Rabies. The DNA sequence is disclosed in Genebank No.:M32751, which is incorporated herein by reference. See also: Anilionis, A., *et al.*, 1981 *Nature* 294:275, which is incorporated herein by reference.

Plasmid pBa.HPV-L1 is a 6.80 kb plasmid which contains a PCR generated fragment encoding the L1 capsid protein of the human papillomavirus (HPV) including HPV strains 16, 18, 31 and 33 cloned into pBabe.puro at the *Bam*HI

and *Eco*RI sites. The plasmid is useful in the immunization against HPV infection and the cancer caused thereby. The DNA sequence is disclosed in Genebank No.:M15781, which is incorporated herein by reference. See also: Howley, P., 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al*. Chapter 58:1625; and Shah, K. and P. Howley, 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al*. Chapter 59; both of which are incorporated herein by reference.

Plasmid pBa.HPV-L2 is a 6.80 kb plasmid which contains a PCR generated fragment encoding the L2 capsid protein of the human papillomavirus (HPV) including HPV strains 16, 18, 31 and 33 cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites. The plasmid is useful in the immunization against HPV infection and the cancer caused thereby. The DNA sequence is disclosed in Genebank No.:M15781, which is incorporated herein by reference. See also: Howley, P., 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al*. Chapter 58:1625; and Shah, K. and P. Howley, 1990 *Fields Virology*, Volume 2, Eds.: Channock, R.M. *et al*. Chapter 59; both of which are incorporated herein by reference.

Plasmid pBa.MNp7 is a 5.24 kb plasmid which contains a PCR generated fragment encoding the p7 coding region including the HIV MN *gag* (core protein) sequence cloned into pBabe.puro at the *Bam*HI site. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated herein by reference.

Plasmid pGA733-2 is a 6.3 kb plasmid that contains the GA733-2 tumor surface antigen cloned from the colorectal carcinoma cell line SW948 into pCDM8 vector (Seed, B. and A. Aruffo, 1987 *Proc. Natl. Acad. Sci. USA* 84:3365, which is incorporated herein by reference) at *Bst*XI site. The tumor-associated target protein is an example of a target protein useful in the immunization against and treatment of

hyperproliferative disease such as cancer. The GA733-2 antigen is a useful target antigen against colon cancer. The GA733 antigen is reported in Szala, S. *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* 87:3542-3546, which is incorporated herein by reference.

Plasmid pT4-pMV7 is a 11.15 kb plasmid that contains cDNA which encodes human CD4 receptor cloned into pMV7 vector at the *Eco*RI site. The CD4 target protein is useful in the immunization against and treatment of T cell lymphoma. Plasmid pT4-pMV7 is available from the AIDS Repository, Catalog No. 158.

Plasmid pDJGA733 is a 5.1 kb plasmid that contains the GA733 tumor surface antigen cloned into pBabe.puro at the *Bam*HI site. The tumor-associated target protein is an example of a target protein useful in the immunization against and treatment of hyperproliferative disease such as cancer. The GA733 antigen is a useful target antigen against colon cancer.

Plasmid pBa.RAS is a 6.8 kb plasmid that contains the ras coding region that was first subcloned from pZIPneoRAS and cloned into pBabe.puro at the *Bam*HI site. The ras target protein is an example of a cytoplasmic signalling molecule. The method of cloning ras is reported in Weinberg 1984 *Mol.. Cell. Biol.* 4:1577, which is incorporated herein by reference. Ras encoding plasmid are useful for the immunization against and treatment of hyperproliferative disease such as cancer; in particular, ras related cancer such as bladder, muscle, lung, brain and bone cancer.

Plasmid pBa.MNp55 is a 6.38 kb plasmid which contains a PCR generated fragment encoding the p55 coding region including the HIV MN gag precursor (core protein) sequence cloned into pBabe.puro at the *Bam*HI site. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated herein by reference.

Plasmid pBa.MNp24 is a 5.78 kb plasmid which contains a PCR generated fragment from the pMN-SF1 template encoding the p24 coding region including the whole HIV MN gag coding region cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pBa.MNp17 is a 5.5 kb plasmid which contains a PCR generated fragment encoding the p17 coding region including the HIV MN *gag* (core protein) sequence cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pBa.SIVenv is a 7.8 kb plasmid which contains a 2.71 PCR generated fragment amplified from a construct containing SIV 239 in pBR322 cloned into pBabe.puro at the *Bam*HI and *Eco*RI sites. The plasmid is available from the AIDS Research and Reference Reagent Program; Catalog No. 210.

Plasmid pcTSP/ATK.env is a 8.92 kb plasmid which contains a PCR generated fragment encoding the complete HTLV envelope coding region from HTLV-1/TSP and /ATK isolates subcloned into the pcDNA1/neo vector. Plasmid pcTSP/ATK.env is reported in 1988 *Proc. Natl. Acad. Sci. USA* 85:3599, which is incorporated herein by reference. The HTLV env target protein is useful in the immunization against and treatment of infection by HTLV and T cell lymphoma.

Plasmid pBa.MNgp160 is a 7.9 kb plasmid which contains a 2.8 kb PCR generated fragment amplified from a

construct containing MNenv in pSP72 and cloned into pBabe.puro at the *BamHI* and *Eco*RI sites. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated herein by reference. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS.

Plasmid pC.MNp55 is a 11.8 kb plasmid which contains a 1.4 kb PCR generated fragment amplified from the *gag* region of MN isolate and cloned into the pCEP4 vector. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M.S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

Plasmid pC.Neu is a 14.2 kb plasmid that contains a 3.8 kb DNA fragment containing the human neu oncogene coding region that was cut out from the LTR-2/erbB-2 construct and subcloned into the pCEP4 vector. The pC.Neu plasmid is reported in DiFiore 1987 *Science* 237:178, which is incorporated herein by reference. The neu oncogene target protein is an example of a growth factor receptor useful as a target protein for the immunization against and treatment of hyperproliferative disease such as cancer; in particular, colon, breast, lung and brain cancer.

Plasmid pC.RAS is a 11.7 kb plasmid that contains a 1.4 kb DNA fragment containing the ras oncogene coding region that was first subcloned from pZIPneoRAS and subcloned into pCEP4 at the BamHI site. The pC.RAS plasmid is reported in Weinberg 1984 *Mol.. Cell. Biol.* 4:1577, which is incorporated herein by reference. The ras target protein is an example of a cytoplasmic signalling molecule. Ras encoding plasmid are useful for the immunization against and treatment of hyperproliferative disease such as cancer; in particular,

ras related cancer such as bladder, muscle, lung, brain and bone cancer.

Plasmid pNLpuro is a 15 kb plasmid which contains HIV *gag/pol* and SV40-puro insertion. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS.

Example 4

It may be important to specifically direct immune responses in order to improve vaccines. The type of immune response (Th1 vs Th2) has been reported to be important in a variety of disease models including infectious diseases, autoimmune diseases, and allergies. To induce strong and stable cell mediated immune response against HIV infection, the use of immunologic adjuvants and immune modulators such as cytokines in conjunction with immunization could enhance cellular immune response and direct antigen-dependent immune response from Th2 to Th1 type.

In order to engineer the immune response *in vivo*, human cytokine genes, either IL-12 genes or GM-CSF genes, were co-delivered along with HIV constructs. Immnune responses induced by co-delivery of these genes with HIV-1 DNA vaccines were examined and the induction of cellular immunity, specifically antiviral CTL responses was studied.

The genes for IL-12 and GM-CSF were individually cloned into expression vectors under control of a CMV promoter. The gene plasmid expression cassettes were then injected into mice along with DNA vaccine cassettes for HIV-1 which have been referred to above or in U.S. Serial Number 08/642,045 filed May 6, 1996, which is incorporated herein by reference). The immunological effects of the co-immunization with these genetic adjuvant cassettes on the magnitude of antigen-specific immune responses was analyzed. A reduction in humoral response was seen with IL-12 co-delivery while a mild enhancement of humoral response was seen with IL-12 co-immunization. An increase in antigen-specific T helper cell proliferation was seen with co-immunization with either IL-12

or GM-CSF. Importantly, a significant CTL induction with co-administration of genes for IL-12 using a direct CTL assay was observed. In contrast, almost no effect on CTL induction was observed with the genes for GM-CSF in these studies. These results demonstrate the utility of DNA vaccines for the tailored production of specific immune responses. They also demonstrate the utility of this approach to elucidate basic immunological functions in a molecule-specific fashion.

MATERIALS AND METHODS

Mice

Balb/c female mice, aged 6-8 weeks were purchased from Harlan Sprague Dawley, Inc., (Indianapolis, Indiana). The mice were housed in a temperature controlled, light-cycled room. Their care were under the guidelines of National Institute of Health and University of Pennsylvania.

Reagents

DNA vaccine formulations pCMN160, pCGN160, pCGag/Pol, were prepared. IL-12 and GM-CSF genes were cloned and inserted into an expression vector with CMV promoter. Recombinant vaccinia (vMN462, vVK1, VV:gag, and vSC8) were obtained from the NIH AIDS Research and Reference Reagent Program.

DNA inoculation

A facilitated DNA inoculation protocol which results in increased *in vivo* protein expression levels from plasmid delivered genes *in vivo* was utilized. Specifically, the quadriceps muscles of BALB/c mice were injected with 100 μl of solution containing 0.25% bupivacaine-HCI (Sigma, St. Louis) using a 27-gauge needle. Two days later, 50 g of the DNA construct of interest in phosphate-saline buffer was injected into the same region of the muscle as the bupivacaine injection. Co-administration of various gene expression cassettes involved mixing the chose plasmids prior to injection.

FACS Analysis

Cells ($1x10^5$) were washed 3x with FACS buffer (PBS containing 1% BSA and 0.1% sodium azide) and incubated with

FITC and/or PE-conjugated mAbs at a saturating conditions for 30 minutes on ice. After being washed 3x with FACS buffer, cells were analyzed using a FACScan (Becton Dickinson).

ELISA

Two μg/ml of gp120 or gp41 (Intracel Corp. Cambridge, MA) in 0.1M carbonate-bicarbonate buffer (pH 9.5) was adsorbed onto microtiter wells overnight at 4°C as previously described. The plate was washed with PBS-0.05% Tween-20 blocked with 3% BSA in PBS with 0.05% Tween-20 for 1 hour at 37°C, then incubated with a manufacturer suggested dilution of HRP-conjugated goat anti-mouse IgG or IgA (Sigma, St Louis, MO). The plate was washed and developed with TM blue buffer (sigma). The OD 450 nm was read on a Dynatech MR5000 plate reader.

T cell Proliferation Assay

Lymphocytes from harvested mouse spleens were prepared. The isolated cell suspensions were resuspended to a concentration of $1x10^6$ cells/mL. A 100 μL aliquot containing $1x10^5$ cells was immediately added to each well of a 96 well microtiter flat bottom plate. Ten μl of protein was added to wells in triplicate of 20 μg/mL. The cells were incubated at 37°C in 5% CO_2 for three days. One μCi of tritiated thymidine was added to each well and the cells were incubated for 12-18 hours at 37°C. The plate was harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). To assure that cells were healthy, 10 g/ml of PHA was used as a polyclonal stimulator positive control.

Cytotoxic T Lymphocyte Assay

A 5 hour chromium^{51} release CTL assay was performed. Lymphocytes were harvested from spleens and prepared as the effector cells by removing the erythrocytes and by washing several times with fresh media. Vaccinia infected targets were prepared by infecting $3x10^6$ p815 cells for 16 hours at 37°C. The target cells were labeled with 100 μCi/ml $Na_2{}^{51}CrO_4$ for 90 min. and used to incubate the stimulated splenocytes for 4-6 hours at 37°C. CTL was tested at effector:target

(E:T) ratio ranging from 50:1 to 12.5:1. Supernatants were harvested and counted on a LKB CliniGamma gamma-counter. Percent specific lysis was determined from the formula:

$$100 \times \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}}$$

Maximum release was determined by lysis of target cells in 1% Triton X-100 containing medium. An assay was not considered valid if the value for the 'spontaneous release' counts are in excess of 20% of the 'maximum release'.

RESULTS

Phenotyping of Mouse Spleens

Following co-inoculation, it was observed that spleens collected from individual experimental groups appeared different. Accordingly, spleens collected from all immunized animals were weighed and visually examined. The spleen weights of these animals are shown in Figure 1A. Whereas the spleens from the mice injected with single formulation controls weighed similar to those of the unimmunized control mice (about 100 mg), the spleens from mince injected with Gag/Pol+IL-12 genes weighed about three times as much as the control spleens. On the other hand, Gag/Pol+GM-CSF immunized mouse spleens were not enlarged. It is interesting to note that those immunized with Gag/Pol only or IL-12 alone did not result in significantly enlarged spleens. Only when the antigen and IL-12 gene cassettes were co-injected did the splenomegaly result, suggesting that this was a combined effect of both gene products. Furthermore, as shown in Figure 1B, the number of lymphocytes derived from the Gag/Pol+IL-12 spleens were more than three times the number derived from the control spleens. Again, the Gag/Pol and GM-CSF immunized mouse spleens did not have any significant increase in the number of lymphocytes above the control spleen cell number. The photograph of representative spleens are shown in Figure 2. Corresponding to their weights, the antigen+IL-12 spleens were observed to be several times larger than other spleens. It has been reported that p35 chain of IL-12 is constitutively

expressed in many cell types. Therefore it would be possible that only a single p40 chain and DNA immunogen were responsible for the effects. To test this, each of the two IL-12 heterodimer genes (p35 and p40) was co-administered with Gag/Pol. As shown in Figure 3, no enlargement of spleen size was observed in either case. These data indicate that the co-injection with DNA vaccine and both p35 and p40 IL-12 genes resulted in the increased size of spleen and corresponding augmentation of the number of splenic cells. These data support that the plasmids entered the same cells in vivo and coordinated transcription of all three components, p35 chain, p40 chain, and the specific antigen, to induce the biological changes observed.

FACS Analysis

To further characterize the cellular composition of the enlarged spleens, FACS analysis was performed. Table 3 shows the FACS results from the double-staining of the splenocytes with antibodies for CD3 with antibodies for B220, CD4, and CD8. As shown, a slight reduction in the percentage of B220 positive B cell population in the groups immunized with Envelop+IL-12 or Gag/Pol+IL-12 constructs (17.46% and 21.62%, respectively) from the percentage of B cell in unimmunized control spleens (25.43%) was observed. In addition, there was a moderate increase in the percentage of CD8+ T cells in the groups immunized with Envelop+IL-12 or Gag/Pol+IL-12 constructs (21.72% and 16.88%, respectively) versus the percentage in the unimmmunized group (13.69%).

Humoral Response

Antisera from immunized mice were collected and analyzed for specific antibody responses against HIV-1 antigens by ELISA. Figure 4 shows the ELISA results from the samples collected at 28 days post-immunization. At 1:100 dilution, sera from the group immunized with pCEnv+pCGM-CSF showed antibody response against HIV-1 gp120 protein which was greater than those of the group immunized with only pCEnv. On the other hand, the group immunized with pCEnv+pCIL-12 showed a significantly less humoral response over the same

period. In repeated experiments, IL-12 generally suppressed specific antibody responses by 1-020% while GM-CSF appeared to have the opposite effect. This humoral effect could be related to the observed change in B cell number in the splenocytes as identified on FACS analysis.

T Cell Proliferation

Activation and proliferation of T helper lymphocytes play a critical role in inducing both humoral immune response via expansion of antigen-activated B cells and cellular immune response via expansion of CD8+ cytotoxic T lymphocytes. Two weeks after DNA immunization, spleens were collected from immunized mice and their lymphocytes were isolated. These cells were then tested for T cell proliferation as described above. Figure 5 shows the proliferation assay results for the mice immunized with DNA vaccine encoding for HIV-1 gag/pol (pCGag/Pol) and those mice co-immunized with pCGag/Pol and IL-12 or GM-CSF. Recombinant p55 protein 20 g/ml of lectin PHA was used as a polyclonal stimulator positive control. As shown, low background level of proliferation was observed from control group from naive mouse spleens with a stimulation index of 1.2 at 1:2 dilution and a moderate level of proliferation was observed from the group immunized with pCGag/Pol alone with a stimulation index of 9.2 at 1:2 dilution. A dramatic boosting in the proliferation was seen from the group co-immunized with pCGag/Pol and IL-12 genes with a stimulation index of 17.1 and the group co-immunized with pCGag/Pol and GM-CSF genes with a stimulation index of 15.6.

CTL Assay Without In Vitro Stimulation

To further investigate the enhancement of the cellular activity, direct CTL assays were conducted. The CTL assay was performed on spleen cells harvested from immunized mice as described above with no in vitro stimulation induced on the splenocytes. The assay was conducted on the day of spleen harvest measuring the chromium release from specific and non-specific vaccinia infected targets. A dramatic increase in specific CTL activity from Gag/Pol+IL-12 immunized

splenocytes (Figure 6) was observed. The control group immunized with only IL-12 gene cassette resulted in no specific lysis of target cells above the background level. In addition, low level (3%) of specific lysis was observed with Gag/Pol only immunization of the 50:1 effector: target ratio. In contrast, 62% specific lysis was seen with Gag/Pol+IL-12 co-administration samples at the 50:1 effector:target ratio and titered out to 9% at the 12.5:1 effector:target ratio. On the other hand, those immunized with Gag/Pol and GM-CSF plasmids resulted in no detectable CTL activity. Similar results were observed from the mice co-immunized with the HIV-1 envelop construct and cytokine genes (Figure 7). The group immunized with Envelop alone and Envelop+GM-CSF resulted in low levels of specific CTL at 4% and 1%, respectively. On the other hand, a dramatically enhancement of CTL activity was observed in the Envelop+IL-12 group at 59% lysis. In both Gag/Pol and Envelope co-immunizations (Figures 6 and 7), the same CTL assay conducted against targets prepared with irrelevant antigen-expressing vaccinia did not result in significant CTL lysis. Therefore, the dramatic enhancement of CTL activity from antigen and IL-12 DNA immunization results were not due to NK activity as the results were antigen specific.

DISCUSSION

The generation of immune responses in vivo using DNA inoculation was reported using different therapeutic targets and delivery techniques.

Induction of cell-mediated immunity may be an important feature for many vaccines. For example, during natural infection, anti-HIV-1 CTL responses appear very early and temporarily appear to correlate with the establishment of the viral set point. CTL T cells play a critical role in viral clearance by targeting and destroying virus-infected cells. Directing immune responses against viral proteins through the development of specific CTL responses would allow induction of a more broad immune response against multiple antigenic targets within the virus. The CTL activity against

the virus is more readily measured in healthy infected patients as compared with AIDS patients, and specific CTL's have been reported to decrease as disease pathogenesis increases clearly linking CTL responses with preferred clinical status. In this regard, a cynomolgous macaque with high specific CTL and low antibody responses was protected against a chimeric SIV/HIV (SHIV) challenge while the animals with low CTL and high antibody responses controlled viral replication but were not completely protected. Specific CTL responses appear to contribute to the maintenance of the asymptomatic phase of HIV-1 infection. Thus the induction of strong HIV-1 specific CTL's in vivo through DNA immunization may play a crucial role in the ultimate protection of the host from the progression of HIV infection.

The potential enhancement of immune responses, especially the CTL response, from DNA vaccines for HIV-1 via co-delivery IL-12 and GM-CSF genes as genetic adjuvants was investigated. The genes for IL-12 and GM-CSF were cloned into expression vectors and injected them into mice along with DNA vaccine cassettes for HIV-1. Co-immunization of plasmids encoding for IL-12 with DNA vaccine for HIV-1 resulted in a dramatic increase in antigen specific CTL response. GM-CSF co-delivery appeared to increase humoral responses while IL-12 co-delivery suppressed humoral response by about 20%.

There are many significant immunological effects of co-delivering cytokine genetic adjuvants with DNA vaccines for HIV-1. First, the size and weight of spleens from mice injected with DNA vaccine and IL-12 genes weighed almost three times as much as the control spleens. In addition, the number of white blood cells from these spleens were more than twice the number of cells from the control spleens. These results agree with previous findings that in vivo administration of recombinant IL-12 in mice caused splenomegaly. Car et al. found that IL-12 injection resulted in a fivefold increase of spleen weight in wild-type mice. These IL-12 induced changes in wild-type mice were associated with markedly increased IFN-gamma serum levels. However, IL-12 administration also

induced a qualitatively similar (to 2 x normal) increase in spleen size in IFN-gamma receptor deficient mice. These earlier studies reported splenomegaly following injection of IL-12 protein. A small amount of IL-12 genes delivered *in vivo* to induce splenomegaly to the level comparable to those published works with recombinant IL-12 proteins. It has been reported that in vivo injection of recombinant IL-12 into mice could have a degree of toxic effects on injected mice such as weight reduction and even death. It is important to note that co-administration of IL-12 genes induced enlarged spleens without any visible adverse changes in the injected mice. This suggests that the likely natural processing and sustained low level delivery through plasmid inoculation may be clinically beneficial. A DNA delivery strategy for inducing significant systemic immune responses is demoinstrated here without toxicity.

Aside from the induction of splenomegaly, the specific immune response from Th2 to Th1 can be manipulated via co-administration of IL-12 genes with DNA vaccines. In this regard, the co-delivery of IL-12 genes with DNA vaccine resulted in the reduction of specific antibody response, while the co-injection of GM-CSF genes resulted in the enhancement of specific antibody response. These results agree with the earlier reportings that IL-12 is a key cytokine in directing the immune response from Th2 to Th1 type response. These antibody results were also in agreement with the spleen cell FACS data where the reduction in the percentage of B220+ B cells were observed with the mice immunized with immunogen (HIV-1 envelop or Gag/Pol) and IL-12 genes. In addition, a significant antigen-specific stimulation of T cells with cytokine co-delivery was observed. The antigen-specific proliferation is a good indicator of CD4 helper T cell induction which appears to be a feature of both cytokines.

To further elucidate the T cell response to the DNA co-immunization, direct CTL assays without *in vitro* stimulation of the harvested splenocytes were conducted. An enhancement of CTL response is a key evidence in demonstrating

the ability to direct immune responses resulting from DNA immunogens from the Th2 to Th1 type response. A dramatic increase in specific CTL response was observed from the group co-immunized with DNA vaccine and IL-12 genes. In summary, genes encoding immunogens and IL-12, a key cytokine responsible producing the Th1 type immune response, were co-adminstered *in vivo* and shown to enhance the cellular immune responses measured by the T cell proliferation and CTL assay.

Co-delivering genes for immunologically important molecules to help direct and manipulate the type and direction of immune responses, for example to direct responses from Th2 to Th1 type, may be used to elicit more clinically efficacious immune responses. By co-administering IL-12 genes with DNA immunogen, humoral responses were moderately suppressed and CTL responses were dramatically increased. In addition splenomegaly, which is characteristic trait in the *in vivo* administration of recombinant proteins IL-12 in mice, was induced. Thus, the power of DNA delivery *in vivo* for both the production of a new generation of more effective vaccines as well as an analytical tool for the molecular dissection of the mechanisms of immune function was demonstrated.

Example 5

To further direct the immune response *in vivo*, the induction and regulation of immune responses from the co-delivery of a broad panel of cytokine genes along with HIV-1 DNA immunogen constructs was investigated. Cytokine gene co-delivery was chosen because cytokines play a critical regulatory and signaling role in immunity. Although cytokines are produced and released by many cells in addition to those of the immune system, cytokines produced by lymphocytes are of a special interest because of their role in regulating cells of the immune system. For instance, the presence IL-2, IFN-γ, and IL-12 activates the T_h0 precursor cell to become a T_h1 inflammatory T cells. On the other hand, the release of IL-4, IL-5, or IL-10 results in a T_h0 precursor becoming an armed T_h2 helper cell. In addition, proinflammatory cytokines

such as IL-1, TNF-α and TNF-β play active roles in the initiation of inflammatory responses.

The role of co-delivery of proinflammatory cytokines (IL-1α, TNF-α, and TNF-β), Th1 cytokines (IL-2, IL-15, and IL-18), and Th2 cytokines (IL-4, IL-5 and IL-10) was investigated. Specifically, genes for these proinflammatory, Th1, and Th2 cytokines were individually cloned into expression vectors under control of a cytomegalovirus (CMV) promoter. The gene plasmid expression cassettes were then injected into mice along with DNA vaccine cassettes for HIV-1 which have been described previously. The immunological effects of the co-injection with these genetic adjuvant cassettes on the direction and magnitude of antigen-specific immune responses were analyzed.

Antigen specific immune responses could be modulated by the co-injection of cytokine genes with DNA immunogen cassettes. More generally, the power of this strategy of co-delivering immunologically important genes as a vehicle for the development of the next generation of DNA vaccines with enhanced potential for clinical efficacy and utility was demonstrated.

MATERIALS AND METHODS

DNA Plasmids

DNA vaccine constructs expressing HIV-1 envelope protein (pCEnv) and gag/pol protein (pCGag/Pol) were prepared using standard techniques and readily available starting materials. The genes for human IL-1α, IL-2, IL-5, IL-10, IL-15, TNF-α, TNF-β and mouse IL-4 and IL-18 were cloned into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA) using standard techniques and readily available starting materials. Human IL-1α, IL-2, IL-5, IL-10, IL-15, TNF-α, and TNF-β have been reported to be active in mouse cells. Plasmid DNA was produced in bacteria and purified using Qiagen Maxi Prep kits (Qiagen, Santa Clara, CA).

Reagents and Cell lines

Human rhabdomyosarcoma (RD) and mouse mastocytoma P815 cell lines were obtained from ATCC (Rockville, MD).

Recombinant vaccinia expressing HIV-1 envelope (vMN462), gag/pol (vVK1), and (-galactosidase (vSC8) were obtained from the NIH AIDS Research and Reference Reagent Program. HIV-1 envelope peptide (RIHIGPGRAFYTTKN) was synthesized using standard techniques and readily available starting materials. Recombinant Pr55 or gp120 protein were obtained from Quality Biological (Gaithersburg, MD). Recombinant p24 protein was purchased from Intracell (Cambridge, MA). Antibodies to IL-1α, IL-2, IL-5, IL-10, IL-15, TNF-α, and TNF-β were obtained from R&D Systems (Minneapolis, MN).

Expression of cytokine gene constructs

Expression of cytokine constructs were verified by immunoprecipitation or cytokine ELISA following transfection into RD cells. The cells were washed twice with PBS, starved for one hour in DMEM lacking serum, methionine and cysteine, and then labeled with 200 μCi/ml (1,200 Ci/mmole) of ^{35}S protein labeling mix (NEN/DuPont). Labeled cells were lysed in 0.5 ml of RIPA buffer (50 mM TrisHCl pH7.6; 150 mM NaCl; 0.2% Triton X-100; 0.2% Deoxycholic acid; 0.1% SDKs and 1 mM PMSF) on ice and then clarified by centrifugation at 15000 r.p.m. for 10 min. The clarified lysates were incubated with relevant antibodies (R&D System) for 90 min. on ice. Protein A sepharose was added to antigen-antibody complexes and mixed by shaking at 4'C for 90 min. The protein pellet was resuspended in 50 μl of 1X sample buffer and heated at 100'C for 3-5 min. after extensive washing in buffers containing high salt and BSA. A fraction of the protein sample was analyzed by SDS 12%-PAGE. For fluorography, gels were soaked in 1M sodium salicylate containing 10% glycerol for 15 min., dried, and autoradiographed using Kodak X-omat-AR film. Supernatants from the transfected RD cells were collected and tested for expression using cytokine ELISA kits (Pharmingen, San Diego, CA and R&D System).

DNA inoculation of mice

The quadriceps muscles of 6 to 8 weeks old balb/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with 50μg of each DNA construct of interest

formulated in phosphate buffered saline (PBS) and 0.25% bupivacaine-HCl (Sigma, St. Louis, MO). Co-administration of various gene expression cassettes involved mixing the chosen plasmids prior to injection for a total of 100 μg per injection.

ELISA

Fifty μl of p24 or gp120 protein diluted in 0.1M carbonate-bicarbonate buffer (pH 9.5) to 2 μg/ml concentration was adsorbed onto microtiter wells overnight at 4°C. The plates were washed with PBS-0.05% Tween-20 and blocked with 3% BSA in PBS with 0.05% Tween-20 for one hour at 37°C. Mouse antisera was diluted with 0.05% Tween-20 and incubated for one hour at 37°C, then incubated with HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). The plates were washed and developed with 3'3'5'5' TMB (Sigma) buffer solution. The plates were read on a Dynatech MR5000 plate reader with the optical density at 450 nm.

T helper cell proliferation assay

Lymphocytes were harvested from spleens and prepared as the effector cells by removing the erythrocytes and by washing several times with fresh media. The isolated cell suspensions were resuspended to a concentration of 5×10^6 cells/ml. A 100 μl aliquot containing 5×10^5 cells was immediately added to each well of a 96 well microtiter flat bottom plate. Recombinant Pr55 or gp120 protein at the final concentration of 5 μg/ml and 1 μg/ml was added to wells in triplicate. The cells were incubated at 37°C in 5% CO_2 for three days. One μCi of tritiated thymidine was added to each well and the cells were incubated for 12 to 18 hours at 37°C. The plate was harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). Stimulation Index was determined from the formula:

Stimulation Index (SI) = (experimental count/spontaneous count)

Spontaneous count wells include 10% fetal calf serum which serves as irrelevant protein control. In addition, pCEnv or control immunized animals routinely have SI of 1 against Pr55 protein. Similarly, pCGag/pol or control routinely have SI of 1 against gp120 protein. To assure that cells were healthy, PHA or con A (Sigma) was used as a polyclonal stimulator positive control. The PHA or con A control samples had a SI of 20-40.

Cytotoxic T lymphocyte assay

A five hour ^{51}Cr release CTL assay was performed using vaccinia infected targets or peptide treated targets. The assay was performed both with and without *in vitro* effector stimulation. In the *in vitro* stimulated assay, the effectors were stimulated with relevant vaccinia-infected cells (vMN462 for envelope and vVK1 for gag/pol), and which were fixed with 0.1% glutaraldehyde or with envelope-specific peptides (RIHIGPGRAFYTTKN) at a 1 (M concentration for four to five days in CTL culture media at 5 x 10^6 cells per ml. CTL culture media consists of 1:1 ratio of Iscove's Modified Dulbecco Media (Gibco-BRL, Grand Island, NY) and Hanks' Balanced Salt Solution (Gibco-BRL) with 10% fetal calf serum 1640 (Gibco-BRL) and 10% RAT-T-STIM without Con A (Becton Dickinson Labware, Bedford, MA). Vaccinia infected targets were prepared by infecting 3 x 10^6 P815 cells at the multiplicity of infection (MOI) of 10-20 for five to twelve hours at 37°C. Peptide treated targets were prepared by incubating P815 cells with 1μM concentration of the peptide. A standard Chromium release assay was performed in which the target cells were labeled with 100 μCi/ml $Na_2{}^{51}CrO_4$ for 60 to 120 minutes and used to incubate with the stimulated effector splenocytes for four to six hours at 37°C. CTL lysis was determined at effector:target (E:T) ratios ranging from 50:1 to 12.5:1. Supernatants were harvested and counted on a LKB CliniGamma gamma-counter. Percent specific lysis was determined from the formula:

$$100 \times \{\underline{\text{experimental release - spontaneous release}}\}$$

maximum release - spontaneous release

Maximum release was determined by lysis of target cells in 1% Triton X-100 containing medium. An assay was not considered valid if the value for the 'spontaneous release' counts are in excess of 20% of the 'maximum release'.

Complement lysis of CD8+ T cells

CD8+ T cells were removed from the splenocytes by a treatment with anti-CD8 monoclonal antibody (Pharmingen, San Diego, CA) followed by incubation with rabbit complement (Sigma) for 45 min. at 37°C.

RESULTS

Expression of cytokine gene cassettes

The cytokine genes were individually cloned into pCDNA3 plasmid expression vectors (Figure 10). These cytokine expression cassettes were verified by a sequencing analysis of the entire insert (on both 5' and 3' sides). In addition, these cytokine genes were transfected *in vitro* into RD cells and the expression of these constructs were verified by immunoprecipitation using relevant antibodies or by cytokine ELISA as described in the Materials and Methods section.

Humoral response following co-injection with cytokine genes

Antisera from pCGag/pol immunized mice were collected and analyzed for specific antibody responses against HIV-1 antigens by ELISA.

In these experiments, 50 μg of each DNA was co-administered intramuscularly at days 0 and 14. Prior to injection and at 28 days following injection, the mice (four mice per group) were bled and the sera were collected. The serial dilutions were 1:100, 1:200, 1:400, 1:800,1:1600, and 1:3200. The background optical density level for ELISA was <0.015. These experiments have been repeated with similar results. As shown in the data, the endpoint antibody titers for these immunized groups were determined using the ELISA against p24 gag protein, and the endpoint antibody titers for these immunized groups were determined using the ELISA against gp120 envelope protein. The following data was generated for

gag/pol-specific antibody titer from sera collected at 28 days post-DNA immunization:

Anti-p24 Antibody Titer	
pCGag/pol Only	400
pCGag/pol + IL-1alpha	1600
pCGag/pol + TNF-alpha	1600
pCGag/pol + TNF-beta	1600
pCGag/pol + IL-2	3200
pCGag/pol + IL-15	800
pCGag/pol + IL-18	3200
pCGag/pol + IL-4	3200
pCGag/pol + IL-5	3200
pCGag/pol + IL-10	1600

The highest level of end point titer was observed with sera from the IL-2, IL-4, IL-5, and IL-18 co-injected groups. A dramatic enhancement of humoral response was also observed with the group co-injected with IL-1α, TNF-α, TNF-β, and IL-10 over the group immunized with pCGag/pol alone. A similar result was seen with the groups immunized with pCEnv.

Anti-gp120 Antibody Titer	
pCEnv Only	200
pCEnv + IL-1alpha	800
pCEnv + TNF-alpha	800
pCEnv + TNF-beta	400
pCEnv + IL-2	800
pCEnv + IL-15	400
pCEnv + IL-18	800
pCEnv + IL-4	1600
pCEnv + IL-5	1600
pCEnv + IL-10	1600

Again, the highest level of end point titer was observed with sera from the IL-4, IL-5, and IL-10 co-injected groups.

Generation of T helper cells

Activation and proliferation of T helper lymphocytes play a critical role in inducing both a humoral immune response via B cells and cellular immune response via CD8+ cytotoxic T cells. Mice received two DNA immunization (50 μg each) separated by two weeks. At one week after the boost injection, the mice were sacrificed, the spleens were harvested, and the lymphocytes were isolated and tested for T helper cell proliferation.

Proinflammatory cytokine co-injection

Proliferation assays were conducted with splenocytes from mice co-injected with pCEnv or pCGag/pol and proinflammatory cytokines IL-1α, TNF-α, and TNF-β.

In the experiments evaluating T helper cell proliferation responses following co-injection with proinflammatory cytokines, IL-1α, TNF-α, and TNF-β the methods were as follows. Two weeks after the first DNA immunization with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested against recombinant gp120 protein (5 and 1 μg/ml final concentrations). Two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for T cell proliferative response against Pr55 protein (5 and 1 μg/ml final concentrations). These experiments have been repeated two times with similar results. The following data was generated:

Antigen Specific T Cell Proliferative Response

	gp120 protein 5μg/ml	gp120 protein 1μg/ml
pCEnv Only	2.2	1.3
pCEnv + IL-1 alpha	2.3	0.1
pCEnv + TNF-alpha	6.1	2.7
pCEnv + TNF-beta	3.1	1.8
Control	0.5	0.2

	p24 protein 5μg/ml	p24 protein 1μg/ml
pCGag/pol Only	2.4	0.8
pCGag/pol + IL-1 alpha	2.8	1.4
pCGag/pol + TNF-alpha	12.4	2.2
pCGag/pol + TNF-beta	4.0	1.9
Control	0.6	0.8

These data show that a background level of proliferation was observed in the control group and a moderate level of proliferation was observed in the group immunized with pCEnv or pCGag/pol alone. Even though the group co-injected with IL-1α did not result in any increase in T helper cell

proliferation in either pCEnv or pCGag/pol immunization, the groups co-injected with pCEnv+TNF-β resulted in a significant enhancement of T helper cell proliferation with the stimulation index of 3.1 at 5 μg/ml gp120 protein concentration. Similarly, pCGag/pol+TNF-β resulted in a stimulation index of 4.0 at 5 μg/ml Pr55 protein concentration. Even higher levels of T helper cell proliferation were observed with pCEnv+TNF-α and pCGag/pol+TNF-α co-injections with stimulation indexes of 6.1 and 12.4, respectively (at 5 μg/ml of each protein concentration).

Th1 cytokine co-injection

The effects of co-delivering Th1 cytokines IL-2, IL-15, and IL-18 was investigated. pCEnv+IL-18 and pCGag/pol+IL-18 co-injection groups resulted in a stimulation indexes of 4.4 and 10.0, respectively (at 5 μg/ml of each protein concentration). In experiments evaluating T helper cell proliferation responses following co-injection with IFN-γ inducing Th1 cytokines, IL-12 and IL-18 the following methods were followed. Two weeks after the first DNA immunization with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested against recombinant gp120 protein (5 and 1 μg/ml final concentrations). Two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for T cell proliferative response against Pr55 protein (5 and 1 μg/ml final concentrations). These experiments have been repeated two times with similar results. The following data was generated

	gp120 protein	
	5μg/ml	1μg/ml
pCEnv Only	2.2	1.3
pCEnv + IL-18	4.4	0.8
pCEnv + IL-12	7.8	3.8
Control	0.5	0.2

	p24 protein 5μg/ml	1μg/ml
pCGag/pol Only	2.4	0.8
pCGag/pol + IL-18	10.0	2.1
pCGag/pol + IL-12	12.0	3.8
Control	0.6	0.8

In addition, pCEnv+IL-2 and pCGag/pol+IL-2 co-injections resulted in stimulation indexes of 6.0 and 12.0, respectively (at 5 μg/ml of each protein concentration). Co-delivery of IL-15, however, resulted in a more moderate increase in T helper cell proliferation. In experiments evaluating T helper cell proliferation responses following co-injection with IL-2 receptor dependent Th1 cytokines, IL-2 and IL-15,, the following methods were used. Two weeks after the first DNA immunization with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested against recombinant gp120 protein (5 and 1 μg/ml final concentrations). Two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for T cell proliferative response against Pr55 protein (5 and 1 μg/ml final concentrations). These experiments have been repeated two times with similar results. The following dfat were generated.

	gp120 protein 5μg/ml	1μg/ml
pCEnv Only	2.2	1.3
pCEnv + IL-2	6.0	2.1
pCEnv + IL-15	2.3	2.0
Control	0.5	0.2

	p24 protein 5μg/ml	1μg/ml
pCGag/pol Only	2.4	0.8
pCGag/pol + IL-2	12.0	2.5
pCGag/pol + IL-15	2.6	0.6
Control	0.6	0.8

Th2 cytokine co-injection

In addition to the examination of proinflammatory and Th1 cytokine co-injection, the effects of co-delivering Th2 cytokines IL-4, IL-5 and IL-10 with pCEnv and pCGag/pol was also investigated. Both the groups co-injected with IL-4 or IL-5 showed a moderate increases in T helper cell proliferation over those of pCEnv or pCGag/pol immunization alone. In experiments evaluating T helper cell proliferation responses following co-injection with Th2 cytokines, IL-5 and IL-10, the following methods were used. Two weeks after the first DNA immunization with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested against recombinant gp120 protein (5 and 1 μg/ml final concentrations). Two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for T cell proliferative response against Pr55 protein (5 and 1 μg/ml final concentrations). These experiments have been repeated two times with similar results. The following data was generated:

	gp120 protein	
	5μg/ml	1μg/ml
pCEnv Only	2.2	1.3
pCEnv + IL-4	3.8	2.9
pCEnv + IL-5	2.4	1.8
pCEnv + IL-10	4.5	2.3
Control	0.5	0.2

	p24 protein	
	5μg/ml	1μg/ml
pCGag/pol Only	2.4	0.8
pCGag/pol + IL-4	5.0	3.1
pCGag/pol + IL-5	3.1	2.1
pCGag/pol + IL-10	8.0	2.4
Control	0.6	0.8

Co-delivery of IL-10 resulted in a more dramatic enhancement of T helper cell proliferation with stimulation indexes of 4.5 and 8.0, respectively (at 5 μg/ml of each protein concentration).

Generation of Cytotoxic T Lymphocytes

To further investigate the enhancement of cellular immunity, cytotoxic T lymphocyte (CTL) assays were performed using splenocytes of mice immunized with pCEnv and pCGag/pol. Mice received two DNA immunization (50 μg each) separated by two weeks. At one week after the boost injection, the mice sacrificed, the spleens harvested, and the lymphocytes were isolated and tested for CTL response. The assay was performed both with and without *in vitro* stimulation of effector splenocytes prior to measuring chromium release from specific and non-specific vaccinia infected or peptide treated targets. To calculate specific lysis of targets, the percent lysis of non-specific (vSC8 infected) targets was subtracted from the percent lysis of specific (vMN462 or vVK1 infected) targets.

CTL Response with *in vitro* stimulation of effectors

Data was generated to evaluate cytotoxic T lymphocyte responses following co-injection with various cytokines. The following data was generated:

Antigen Specific CTL Response

	50:1	25:1	12.5:1
pCEnv	10%	4%	3%
pCEnv + IL-1 alpha	14%	10%	6%
pCEnv + TNF-alpha	30%	23%	18%
pCEnv + TNF-beta	20%	16%	13%
pCEnv + IL-2	22%	16%	4%
pCEnv + IL-15	46%	28%	10%
pCEnv + IL-12	35%	24%	19%
pCEnv + IL-18	22%	16%	13%
pCEnv + IL-4	4%	4%	7%
pCEnv + IL-5	13%	11%	9%
pCEnv + IL-10	13%	8%	3%
Control	3%	2.0%	3.5%

	50:1	25:1	12.5:1
pCGag/pol	12%	11%	7%
pCGag/pol + IL-1α	16%	8%	1%
pCGag/pol + TNF-α	29%	20%	7%
pCGag/pol + TNF-β	12%	13%	3%
pCGag/pol + IL-2	14%	11%	10%
pCGag/pol + IL-15	30%	21%	7%
pCGag/pol + IL-12	38%	24%	15%
pCGag/pol + IL-18	19%	15%	4%
pCGag/pol + IL-4	2%	2%	2%
pCGag/pol + IL-5	0%	3%	2%
pCGag/pol + IL-10	6%	6%	0%
Control	3.3%	2.8%	4.5%

In experiments performed evaluating cytotoxic T lymphocyte responses following co-injection with proinflammatory cytokines, IL-1α, TNF-α, and TNF-β, two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vVK1) and non-specific vaccinia (vSC8). Two weeks after the first DNA co-injection with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vMN462) and non-specific vaccinia (vSC8). These experiments have been repeated two times with similar results.

In experiments performed evaluating cytotoxic T lymphocyte responses following co-injection with IFN-γ inducing Th1 cytokines, IL-12 and IL-18, two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vVK1) and non-specific vaccinia (vSC8). Two weeks after the first DNA co-injection with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vMN462) and non-specific vaccinia (vSC8). These experiments have been repeated two times with similar results.

In experiments performed evaluating cytotoxic T lymphocyte responses following co-injection with IL-2 receptor dependent Th1 cytokines, IL-2 and IL-15, two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage.

After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vVK1) and non-specific vaccinia (vSC8). Two weeks after the first DNA co-injection with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vMN462) and non-specific vaccinia (vSC8). These experiments have been repeated two times with similar results.

In experiments performed evaluating cytotoxic T lymphocyte responses following co-injection with Th2 cytokines, IL-5 and IL-10, two weeks after the first DNA co-injection with pCGag/pol (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vVK1) and non-specific vaccinia (vSC8). Two weeks after the first DNA co-injection with pCEnv (50 μg of each), the mice (four mice per group) were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated and tested for CTL response using target cells infected with specific (vMN462) and non-specific vaccinia (vSC8). These experiments have been repeated two times with similar results.

Proinflammatory cytokine co-injection

With regard to data from the CTL assay results for the mice co-injected with pCEnv or pCGag/pol and proinflammatory cytokines IL-1α, TNF-α, and TNF-β, a background level of specific killing was observed from the control animals, whereas the animals immunized with pCEnv alone showed a small level of CTL response. Co-injection with pCEnv+IL-1α or pCEnv+TNF-β resulted in a moderate increase in CTL activity. On the other hand, a more dramatic increase in the specific killing of targets infected with vaccinia

(vMN462) expressing HIV-1 envelope was observed after co-injection with pCEnv+TNF-α. Greater than 30% specific lysis of target cells was observed after co-injection with pCEnv+TNF-α at a 50:1 effector to target (E:T) ratio. Similarly, the mice immunized with pCGag/pol+TNF-α resulted in a significant enhancement of antigen-specific CTL lysis of targets infected with vaccinia (vVK1) expressing HIV-1 gag/pol (29% lysis at E:T ratio of 50:1) while co-injection with pCGag/pol+IL-1α or pCGag/pol+TNF-β resulted in a small increase in CTL response.

Th1 cytokine co-injection

The effects of co-delivering Th1 cytokines with DNA vaccine constructs was investigated. With regard to CTL assay results for the mice immunized with pCEnv and those mice co-injected with Th1 cytokines IL-12 and IL-18. Unlike IL-12 co-administration, IL-18 co-injection resulted in a more moderate increase in CTL response. Co-administration of IL-2 also resulted in a moderate increase in CTL response. On the other hand, a more dramatic increase in CTL response at 46% specific lysis was observed after pCEnv+IL-15 immunization. Similarly, the mice injected with pCGag/pol+IL-15 resulted in a significant enhancement of antigen-specific CTL lysis (at 30%).

Th2 cytokine co-injection

In addition to investigating the effects from the co-delivery of proinflammatory and Th1 cytokines, the effects of co-injections with Th2 cytokines IL-4, IL-5 and IL-10 on the level of CTL response were also studied. Although the co-injections with these cytokines resulted in the increase in T helper cell proliferative responses, the co-injections of IL-4, IL-5 or IL-10 with either pCEnv or pCGag/pol did not result in any specific increase in CTL response.

Determination of MHC class I restriction in CTL response

To determine whether the increases in CTL response via co-injection with TNF-α and IL-15 was due to a CD8+ MHC class I restricted stimulus, CTL assays were performed using a HIV-1 envelope peptide (RIHIGPGRAFYTTKN) which has been

shown to be a specific epitope for MHC class I-restricted CTL for balb/c mice. Mice received two immunizations of 50 μg of each DNA construct separated by two weeks and their spleens were harvested one week after the second immunization. The CTL assay was performed on the splenocytes following *in vitro* stimulation with envelope-specific peptides. A significant enhancement of CTL response was observed after both co-injection (Figure 11A) with IL-15 and TNF-α at 25% and 32% specific killing at an E:T ratio of 50:1, respectively. This observation was verified by measuring CTL activity after the removal of CD8+ T cells from the effector cell population by complement lysis. Mice received two immunizations of 50 μg of each plasmid at the same interval as above. A CTL assay was performed in which one group of effector cells was treated as before and CD8+ T cells from the second group were removed. As shown in Figure 11B, the removal of CD8+ T cells resulted in the suppression of antigen-specific CTL enhancement observed after co-injections with IL-15 and TNF-α. These results indicate that the enhancement of cytolytic activity was antigen-specific, class I-restricted and CD8+ T cell dependent.

Direct CTL response (without *in vitro* stimulation of effectors)

The level of direct CTL response induced by co-injection with TNF-α or IL-15 was investigated because a high and consistent level of CTL response (with *in vitro* stimulation) was observed from these two co-administration groups. The chromium release assay was performed on the same day the splenocytes were isolated. Unlike seeing direct CTL with IL-12 co-injection, the induction of direct CTL activity after co-injection with pCEnv and TNF-α or IL-15 (Figure 12) was not observed.

DISCUSSION

The overall aim of any immunization strategy is to induce potent and durable pathogen-specific immune responses using the least number of immunizations. However, the correlates of protection from may vary from one pathogen to

the next and improvements in results can be achieved by directing the immune response. For example, high levels of specific antibody response are thought to be important for protection from infection with hepatitis B virus while protection from lymphocytic choriomeningitis virus (LCMV) infection is mediated primarily through T cell-mediated responses. The design of DNA vaccination strategies can be improved by tailoring the direction and magnitude of induced immune responses to fit the correlates of protection for each target pathogen.

As a new immunization strategy, nucleic acid immunization has been demonstrated to elicit both antigen-specific humoral and cellular immune responses *in vivo* in a variety of animal models. More clinically efficacious vaccines may be produced using a strategy of controlling the direction and magnitude of immune responses. The finer control in generating specific types and directions of the immune responses from vaccine and immune therapies can be accomplished by the co-delivery of genes for immunologically important molecules such as cytokines and costimulatory molecules.

Cytokines play important roles in the immune and inflammatory responses as the initiators and regulators of the immune network. Based upon their specific function in the immune system these cytokines could be further grouped as proinflammatory, Th1, and Th2 cytokines. Proinflammatory cytokines IL-1, TNF-α and TNF-β play important role as the initiator of the host responses to injury and infection. IL-1 indirectly activates T cells by inducing the production of IL-2 and up-regulating the IL-2R on these cells. IL-1 also influences B cells by inducing their differentiation, growth, and synthesis of IgGs. At least two forms of IL-1, designated as IL-1α and IL-1β, exist and exhibit similar activities. TNF-α and TNF-β are closely related proteins (about 30% amino acid residue homology) which bind to the same cell surface receptors. TNF-α is produced by activated macrophages and monocytes neutrophils, activated lymphocytes, and NK cells

whereas TNF-β is produced by lymphocytes. TNF-α and TNF-β are also implicated in septic shock following infection by Gram-negative bacteria and in rheumatoid arthritis. Furthermore, TNF-α has been suggested to play a pivotal role in regulating the synthesis of other proinflammatory cytokines.

Th1 cytokines regulate the cellular or T cell-mediated arm of the immune response. IFN-γ, a prototypical Th1-type cytokine, is produced by Th1, CD8+, and NK cells, and has been shown to have antiviral effects as well as immunomodulatory effects such as the up-regulation of MHC class I and II antigens. A new cytokine IFN-γ-inducing factor (IGIF) or IL-18 has been found to enhance the production of IFN-γ while inhibiting the production of IL-10 in stimulated PBMC. IL-18 also augments natural killer (NK) cell activity in cultures of human peripheral blood mononuclear cells (PBMC), similar to the structurally unrelated cytokine IL-12. IL-2 is produced primarily by T cells activated by external stimulation; it is critical for the proliferation and clonal expansion of antigen-specific T cells. IL-2 serves this pivotal role in T cell activation by its interaction with a receptor system consisting of three chains, α, β, and γc chains . IL-15, a newly identified homologue of IL-2, is a pleiotropic cytokine which possesses T cell stimulatory activities similar to IL-2.

Th2 cytokines regulate the humoral or antibody-mediated arm of the immune response. IL-5 is a dimeric cytokine that controls the differentiation of B cells into antibody producing plasma cells. IL-5 has been shown to induce antigen-specific IgA production by murine and human B cell. In addition, IL-5 also promotes the growth and proliferation of eosinophils. Although IL-10 has been shown initially to be produced by Th2 T cell clones, it is also produced by B cells and monocytes. Touted as a prototypical Th2-type cytokine, IL-10 has been shown to inhibit the production of such cytokines as IL-1α, IL-6, IL-8, and TNF-α by mitogen-activated monocytes as well as to inhibit the

macrophage activating effects of IFN-γ. A possible role of IL-10 in HIV-1 infection has also been reported; IL-10 mRNA was up-regulated and increased levels of IL-10 were observed from PBMC from asymptomatic HIV-positive individuals compared with PBMC from uninfected individuals. In addition, IL-10 has been shown to decrease in vitro viral replication in human macrophages.

Expression cassettes for proinflammatory, Th1, and Th2 cytokines were developed in an effort to analyze their ability to function as *in vivo* modulators of the immune responses induced by DNA vaccines. Cytokine genes were co-delivered along with DNA immunogen constructs into mice intramuscularly and analyzed their effects in the direction and magnitude of induced immune responses. A dramatic increase in the antibody response was observed with co-injection with IL-2, IL-4, IL-5, IL-10, and IL-18. Co-injection with TNF-α, TNF-β, IL-2, IL-10, and IL-18 resulted in a dramatic enhancement of T helper proliferation response while co-injection with IL-5 and IL-15 resulted in a more moderate increase in T helper proliferation. Furthermore, among all co-injection combinations, only TNF-α and IL-15 co-injections resulted in a level of CTL enhancement (greater than 30% specific lysis) similar to that of IL-12 co-injection. Co-injection with TNF-β, IL-2, and IL-18 resulted in a more moderate increase in CTL response over those groups immunized with only DNA immunogen. As observed with IL-12 or CD86 co-injection, the enhancement of CTL responses observed from the co-injections with TNF-α and IL-15 were restricted by MHC Class I and CD8+ T cells.

IL-18 has been reported to share similar activities of IL-12. For instance, IL-18 augments natural killer (NK) cell activity in cultures of human peripheral blood mononuclear cells (PBMC), similarly to the structurally unrelated cytokine IL-12. IL-18 also enhances the production of IFN-γ while inhibiting the production of IL-10 in concanavalin A (Con A)-stimulated PBMC. IL-18 has been observed to induce IFN-(through IL-12-independent pathway.

Even though a significant increase in the level of T cell mediated responses and a moderate reduction in humoral response with IL-12 co-delivery, similar effects from IL-18 co-administration was not observed. Instead, a significant increase in the antibody titers with IL-18 co-administration was seen. In addition, unlike IL-12 co-delivery which induced a dramatic enhancement of CTL, but IL-18 co-injection did not induce a similar level of CTL enhancement. The immunomodulatory characteristics of IL-18 appeared to be similar to that of IL-10.

In addition to the differential *in vivo* effects of IL-12 and IL-18 co-administrations, co-injection with IL-2 and IL-15 also resulted in different direction and magnitude of immune responses. IL-2 and IL-15 have been reported to have similar bioactivities which include the sharing of the γ chain of the IL-2 receptor and signaling machinery for T cell stimulation. IL-2 co-administration resulted in a dramatic increase in antibody and T helper cell proliferative responses while the IL-15 co-injection resulted in a significant enhancement of CTL responses. Such differences may be explained by the pleiotropic nature of IL-15. For example, IL-15 has been reported to induce significant TNF-α production in rheumatoid arthritis through activation of synovial T cells. On the other hand, IL-2 induced significantly lower level of TNF-α. This *in vivo* data suggest that signaling machinery is differentially activated by engagement of these two molecules.

Th2 cytokines could be used to improve Th2-type immune responses without affecting the level of T cell-mediated responses. IL-4, IL-5 and IL-10 have been reported to be potent Th2 cytokines. A significant increase in the level of antibody response as well as the level of T helper proliferation with IL-4, IL-5 and IL-10 co-delivery. On the other hand, no increase in the level of CTL response was observed. These results demonstrate that a Th2-type response could be engineered with IL-4, IL-5, or IL-10 co-administration.

A dramatic observation was the role of TNF-α and IL-15 as multi-functional immune modulators.

The role of various immunologically important cytokines on the induction of host immune responses from DNA immunization was investigated. As summarized in Figure 13, the induction of specific arms of immune responses could be engineered using the strategy of co-administration of cytokine genes. This cytokine gene adjuvant network underscores a new level of control in the induction of specific immune responses to tailor the vaccination programs more closely to the correlates of protection which vary from disease to disease. This type of fine control of vaccine and immune therapies was previously unattainable. As a result, controlling the magnitude and direction of the immune response could be advantageous in a wide variety of vaccine strategies. For instance, in a case where T cell mediated response is paramount, but the humoral response may not be needed or even be harmful, IL-12 genes could be chosen as the immune modulator to be co-delivered with a specific DNA immunogen. On the other hand, for building vaccines to target extracellular bacteria, for example, IL-4, IL-5 or IL-10 genes could be co-injected. Furthermore, in cases where both CD4+ T helper cells and antibodies play more important roles in protection, GM-CSF as well as IL-2 could be co-delivered. Lastly, in cases where all three arms of immune responses are critical, TNF-α could be co-injected to give a combined enhancement of antibody, T helper cell, and CTL responses.

Example 6

Using PCR reactions an insert, designated as BL1 and shown in Figure 14, was cloned and ligated it into PCR3 eukaryotic expression vector as well as the vector pBBKan using the appropriate restriction enzymes as shown in Figure 15. The BL1 construct was co-admministered with different HIV-1 antigens and measured the immunostimulatory effects in mice. The results, which are presented in Figures 16 and 17A, 17B, 17C and 17D indicate that the DNA fragment in BL1 enhances

immune responses when coimmunized with HIV-1 antigens. The different observed effects are the increase in spleen size, and an increase in antibody and CTL response.

Table 1

Picornavirus Family

Genera: Rhinoviruses: (Medical) responsible for ~ 50% cases of the common cold.
Etheroviruses: (Medical) includes polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus.
Apthoviruses: (Veterinary) these are the foot and mouth disease viruses.
Target antigens: VP1, VP2, VP3, VP4, VPG

Calcivirus Family

Genera: Norwalk Group of Viruses: (Medical) these viruses are an important causative agent of epidemic gastroenteritis.

Togavirus Family

Genera: Alphaviruses: (Medical and Veterinary) examples include Senilis viruses, RossRiver virus and Eastern & Western Equine encephalitis.
Reovirus: (Medical) Rubella virus.

Flariviridue Family

Examples include: (Medical) dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses.

Hepatitis C Virus: (Medical) these viruses are not placed in a family yet but are believed to be either a togavirus or a flavivirus. Most similarity is with togavirus family.

Coronavirus Family: (Medical and Veterinary)
Infectious bronchitis virus (poultry)
Porcine transmissible gastroenteric virus (pig)
Porcine hemagglutinating encephalomyelitis virus (pig)
Feline infectious peritonitis virus (cats)
Feline enteric coronavirus (cat)
Canine coronavirus (dog)
The human respiratory coronaviruses cause ~40 cases of common cold. EX. 224E, 0C43
Note - coronaviruses may cause non-A, B or C hepatitis

Target antigens:
E1 - also called M or matrix protein
E2 - also called S or Spike protein
E3 - also called HE or hemagglutin-elterose glycoprotein (not present in all coronaviruses)
N - nucleocapsid

Rhabdovirus Family
Genera: Vesiliovirus
Lyssavirus: (medical and veterinary) rabies
Target antigen:
G protein
N protein

Filoviridue Family: (Medical)
Hemorrhagic fever viruses such as Marburg and Ebola virus

Paramyxovirus Family:
Genera: Paramyxovirus: (Medical and Veterinary) Mumps virus, New Castle disease virus (important pathogen in chickens)
Morbillivirus: (Medical and Veterinary) Measles, canine distemper
Pneuminvirus: (Medical and Veterinary) Respiratory syncytial virus

Orthomyxovirus Family (Medical)
The Influenza virus

Bungavirus Family
Genera: Bungavirus: (Medical) California encephalitis, LA Crosse
Phlebovirus: (Medical) Rift Valley Fever
Hantavirus: Puremala is a hemahagin fever virus
Nairvirus (Veterinary) Nairobi sheep disease
Also many unassigned bungaviruses

Arenavirus Family (Medical)
LCM, Lassa fever virus

Reovirus Family
Genera: Reovirus: a possible human pathogen
Rotavirus: acute gastroenteritis in children
Orbiviruses: (Medical and Veterinary) Colorado Tick fever, Lebombo (humans) equine encephalosis, blue tongue

Retrovirus Family
Sub-Family:
Oncorivirinal: (Veterinary) (Medical) feline leukemia virus, HTLVI and HTLVII
Lentivirinal: (Medical and Veterinary) HIV, feline immunodeficiency virus, equine infections, anemia virus
Spumavirinal

Papovavirus Family
Sub-Family:

Polyomaviruses: (Medical) BKU and JCU viruses

Sub-Family:

Papillomavirus: (Medical) many viral types associated with cancers or malignant progression of papilloma

Adenovirus (Medical)

EX AD7, ARD., O.B. - cause respiratory disease - some adenoviruses such as 275 cause enteritis

Parvovirus Family (Veterinary)

Feline parvovirus: causes feline enteritis
Feline panleucopeniavirus
Canine parvovirus
Porcine parvovirus

Herpesvirus Family

Sub-Family: alphaherpesviridue
Genera: Simplexvirus (Medical)
HSVI, HSVII
Varicellovirus: (Medical - Veterinary)
pseudorabies - varicella zoster

Sub-Family - betaherpesviridue
Genera: Cytomegalovirus (Medical)
HCMV
Muromegalovirus

Sub-Family: Gammaherpesviridue
Genera: Lymphocryptovirus (Medical)
EBV - (Burkitts lympho)
Rhadinovirus

Poxvirus Family

Sub-Family: Chordopoxviridue (Medical - Veterinary)
Genera: Variola (Smallpox)
Vaccinia (Cowpox)
Parapoxivirus - Veterinary
Auipoxvirus - Veterinary
Capripoxvirus
Leporipoxvirus
Suipoxvirus

Sub-Family: Entemopoxviridue

Hepadnavirus Family

Hepatitis B virus

Unclassified

Hepatitis delta virus

Table 2

Bacterial pathogens

Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal. Pathogenic gram-negative cocci include: meningococcal; and gonococcal.

Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigellosis; hemophilus; chancroid; brucellosis; tularemia; yersinia (pasteurella); streptobacillus moniliformis and spirillum ; listeria monocytogenes; erysipelothrix rhusiopathiae; diphtheria; cholera; anthrax; donovanosis (granuloma inguinale); and bartonellosis.

Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidiodomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis.

Rickettsial infections include rickettsial and rickettsioses.

Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections.

Pathogenic eukaryotes

Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

Claims

1. A plasmid comprising a nucleotide sequence that encodes

a) an immunomodulating protein selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1 operably linked to regulatory elements and

b) a nucleotide sequence that encodes an immunogen.

2. The plasmid of claim 1 wherein said immunogen is a target protein operably linked to regulatory elements, wherein the target protein encodes a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

3. The plasmid of claim 1 wherein said immunogen is an HIV-1 antigen.

4. The plasmid of claim 1 wherein said immunomodulatory protein is a single chain IL-12.

5. The plasmid of claim 1 comprising nucleotide sequences that encode a plurality of an immunomodulating proteins selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1, wherein each of said nucleotide sequences that encode an immunomodulating protein is operably linked to regulatory elements.

6. The plasmid of claim 1 comprising a plurality of nucleotide sequences that encode said immunomodulating protein.

7. A pharmaceutical composition comprising the plasmid of claim 1.

8. A method of immunizing an individual against a pathogen comprising administering to said individual a plasmid of claim 1.

9. A composition comprising two or more plasmids including:

a first plasmid comprising a nucleotide sequence that encodes an immunomodulating protein selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1 operably linked to regulatory elements and

a second plasmid comprising a nucleotide sequence that encodes an immunogen.

10. The composition of claim 9 where said second plasmid encodes an immunogen selected from the group consisting of a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

11. The composition of claim 9 wherein said immunogen is an HIV-1 antigen.

12. The composition of claim 9 wherein said immunomodulatory protein is a single chain IL-12.

13. The composition of claim 9 wherein said first plasmid comprises nucleotide sequences that encode a plurality of an immunomodulating proteins selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1, wherein each of said nucleotide sequences that encode an immunomodulating protein is operably linked to regulatory elements.

14. The composition of claim 9 comprising a third plasmid, wherein said third plasmid comprises a nucleotide sequence that encodes an immunomodulating protein selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α,

TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1 operably linked to regulatory elements.

15. The composition of claim 9 wherein said first plasmid comprises a plurality of nucleotide sequences that encode said immunomodulating protein.

16. A pharmaceutical composition comprising the composition of of claim 9.

17. A method of immunizing an individual against a pathogen comprising administering to said individual a composition of claim 9.

18. A recombinant vaccine comprising a nucleotide sequence that encodes an immunomodulating protein selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1 operably linked to regulatory elements and a nucleotide sequence that encodes an immunogen.

19. The recombinant vaccine of claim 18 wherein said immunogen is selected from the group consisting of: a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

20. The recombinant vaccine of claim 18 wherein said vaccine is a recombinant vaccinia vaccine.

21. The recombinant vaccine of claim 18 wherein said immunomodulatory protein is a single chain IL-12.

22. The recombinant vaccine of claim 18 wherein said immunogen is a pathogen antigen.

23. The recombinant vaccine of claim 18 comprising nucleotide sequences that encode a plurality of an

immunomodulating proteins selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1, wherein each of said nucleotide sequences that encode an immunomodulating protein is operably linked to regulatory elements.

24. The recombinant vaccine of claim 18 comprising a plurality of nucleotide sequences that encode said immunomodulating protein.

25. A method of immunizing an individual against a pathogen comprising administering to said individual a recombinant vaccine of claim 18.

26. A live attenuated pathogen comprising a nucleotide sequence that encodes an immunomodulating protein selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1 operably linked to regulatory elements.

27. The live attenuated pathogen of claim 26 comprising nucleotide sequences that encode a plurality of an immunomodulating proteins selected from the group consisting of IL-12, GM-CSF, IL-1, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL-10, IL-15, IL-18, and BL-1, wherein each of said nucleotide sequences that encode an immunomodulating protein is operably linked to regulatory elements.

28. The live attenuated pathogen of claim 26 comprising a plurality of nucleotide sequences that encode said immunomodulating protein.

29. A method of immunizing an individual against a pathogen comprising administering to said individual the live attenuated pathogen of claim 26.

30. A plasmid comprising a nucleotide sequence that encodes single chain IL-12.

31. A substantially pure BL-1 protein having an amino acid sequence set forth in Figure 14, or an immunomodulatory fragment thereof.

32. A recombinant expression vector comprising a nucleic acid sequence that encodes a protein of claim 21.

33. The recombinant expression vector of claim 22 comprising the nucleotide sequence in Figure 14.

34. An isolated antibody which binds to an epitope on a protein of claim 21.

35. The antibody of claim 24 wherein said antibody is a monoclonal antibody.

36. A pharmaceutical composition comprising a nucleic acid molecule of claim 22 and a pharmaceutically acceptable carrier.

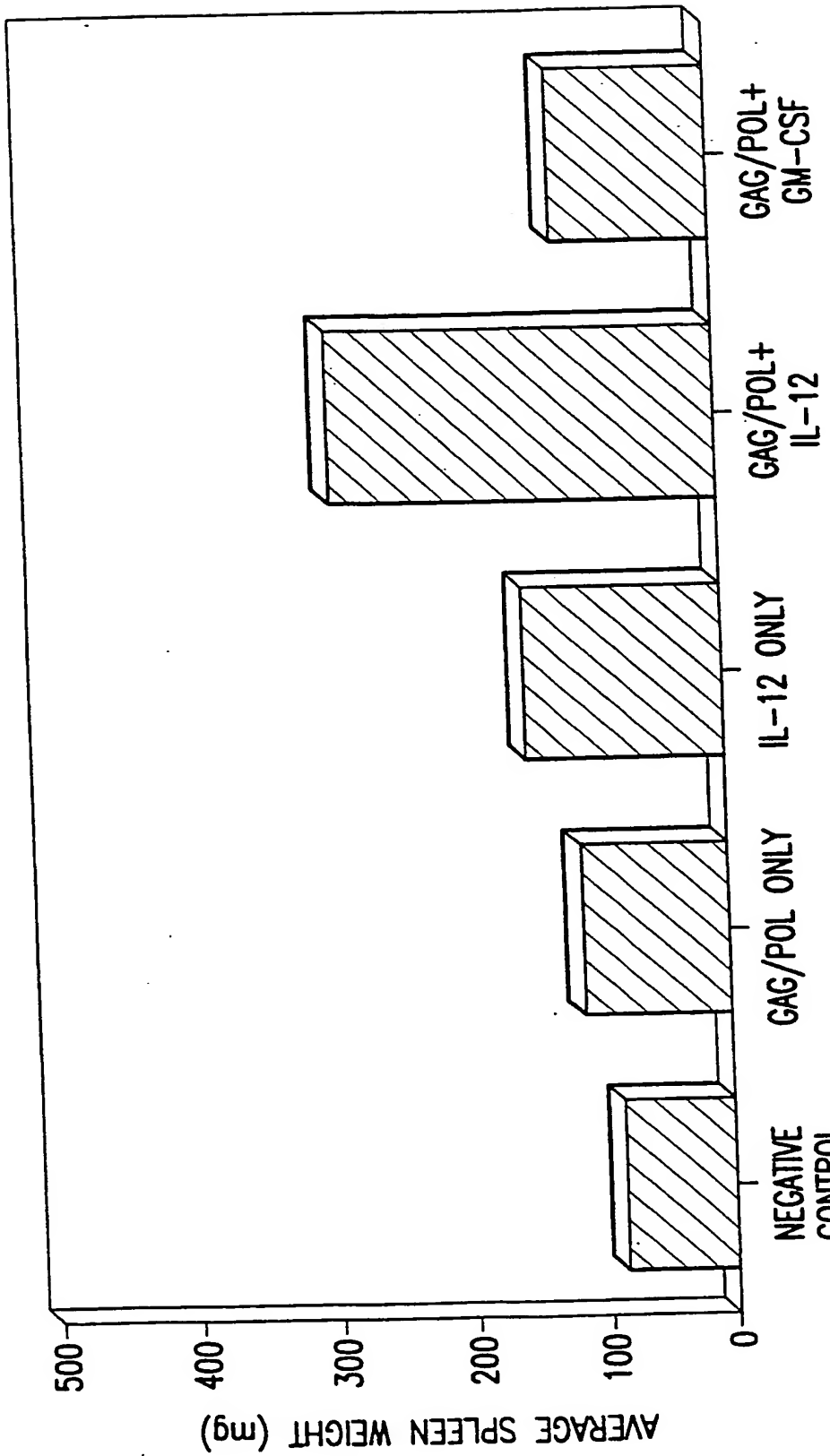


FIG. 1A

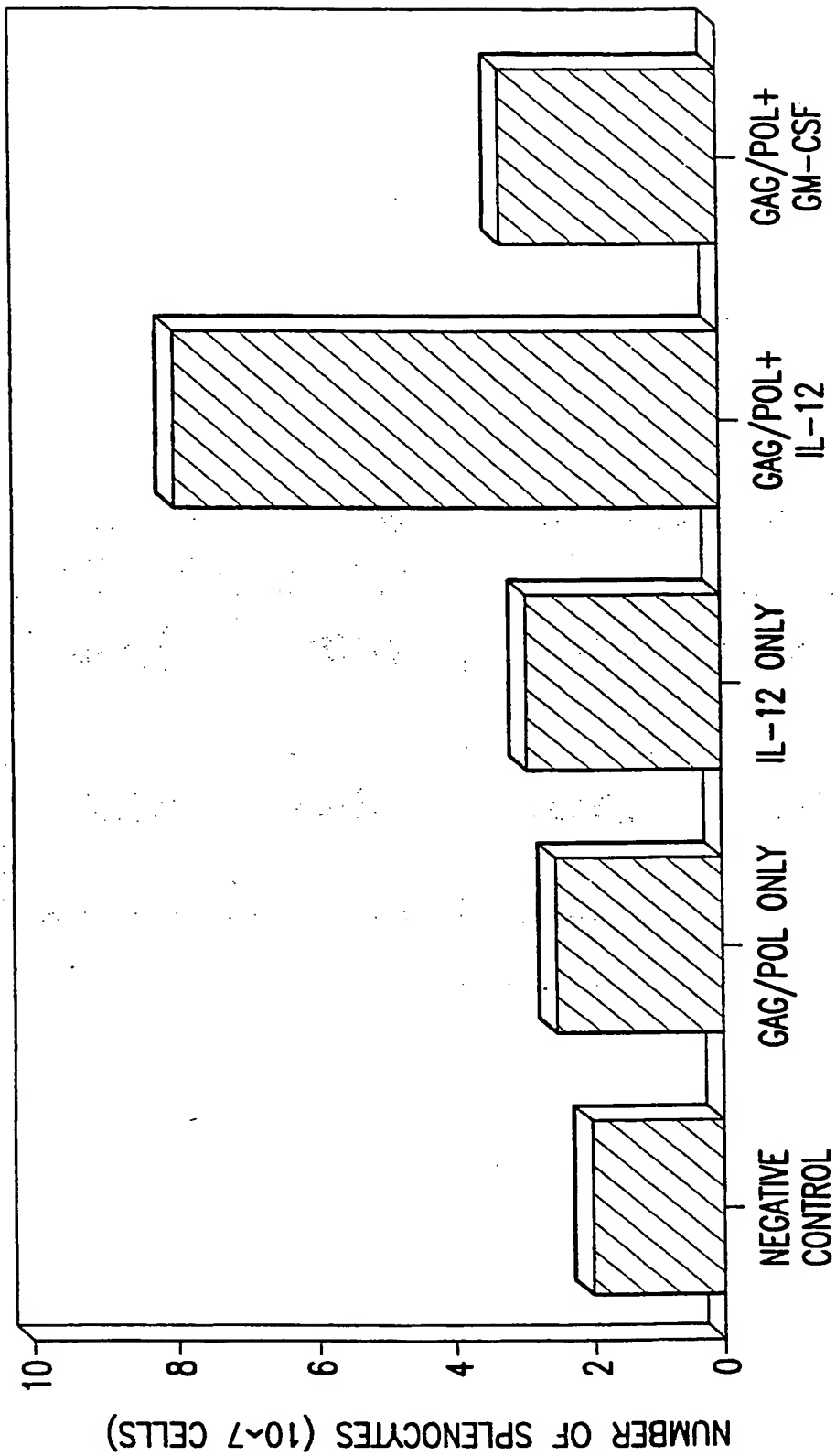


FIG.1B

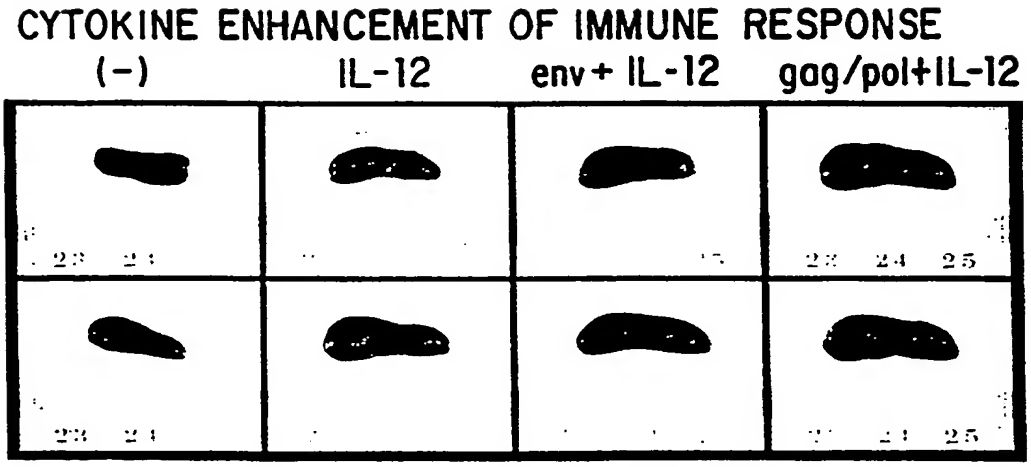


FIG. 2

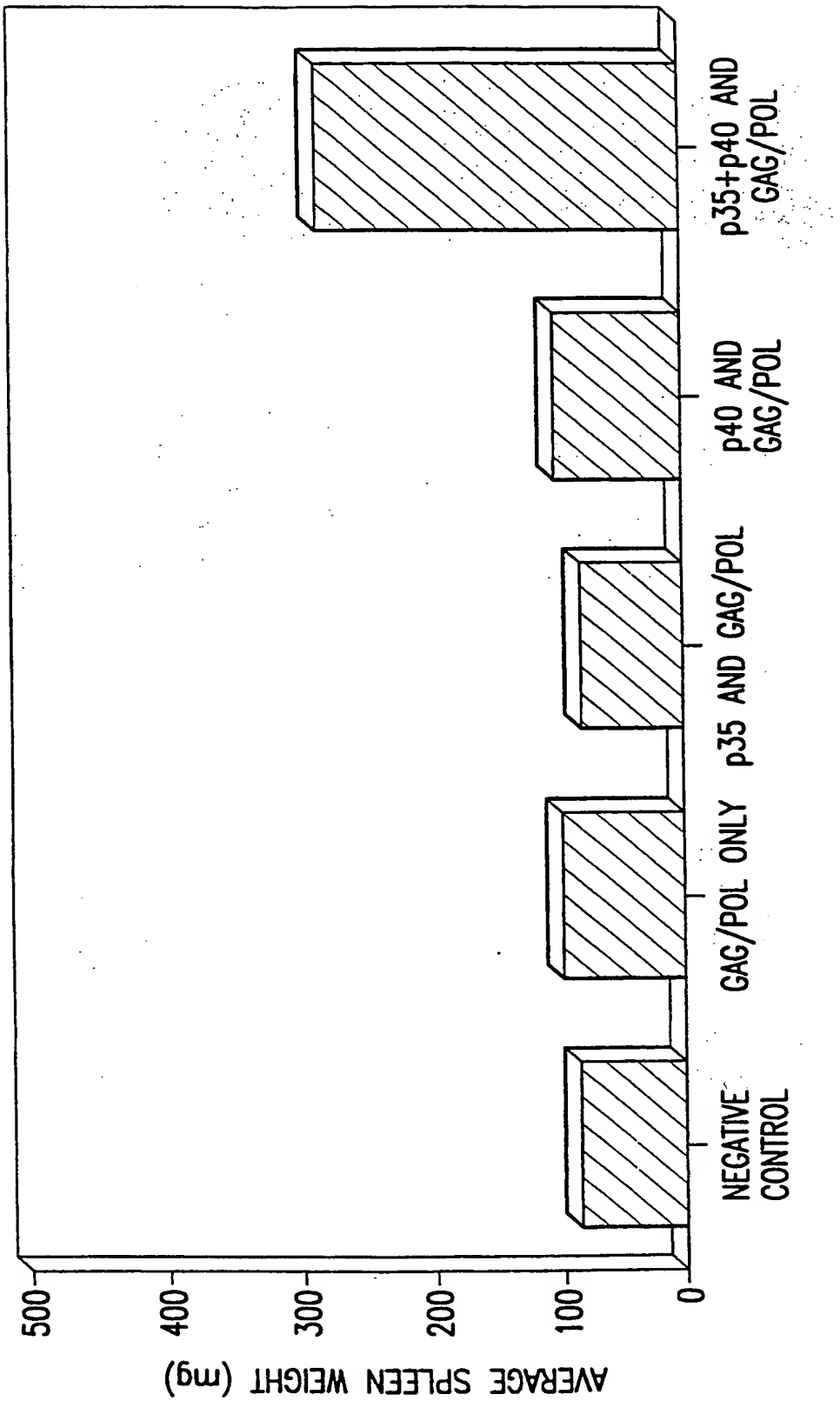


FIG.3

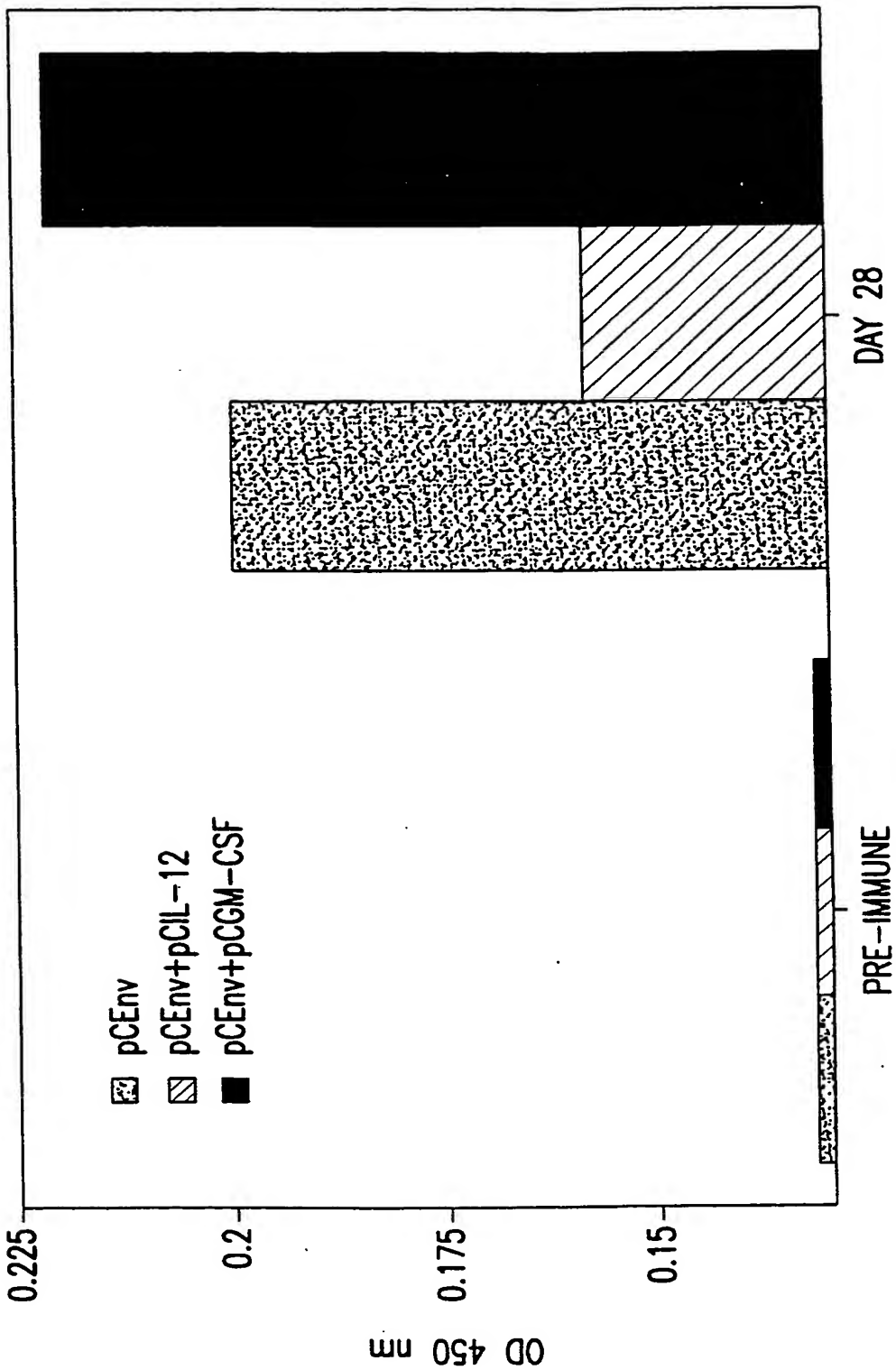


FIG.4

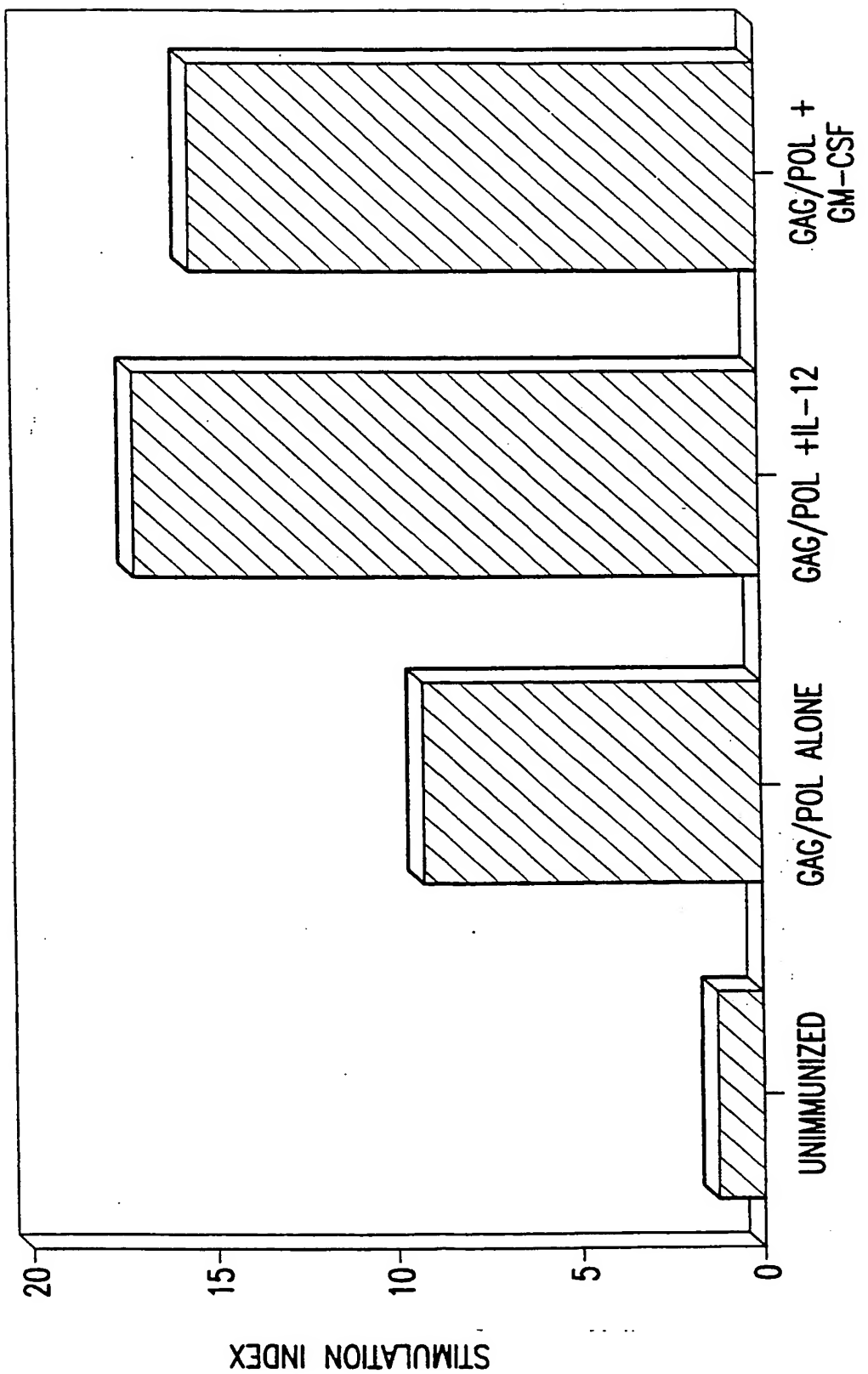


FIG.5

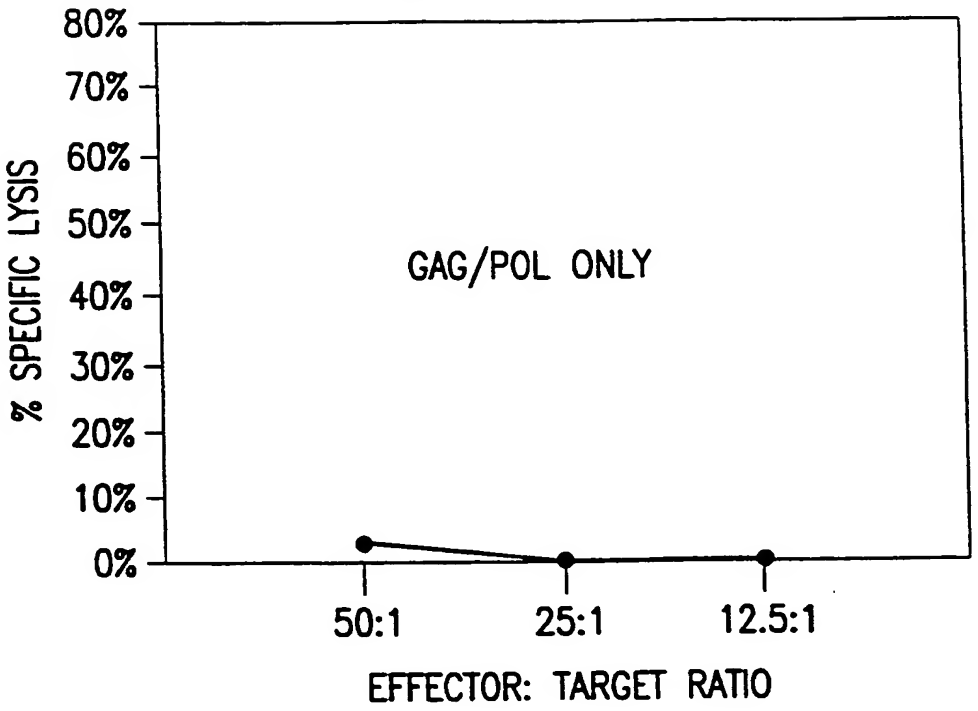


FIG.6A

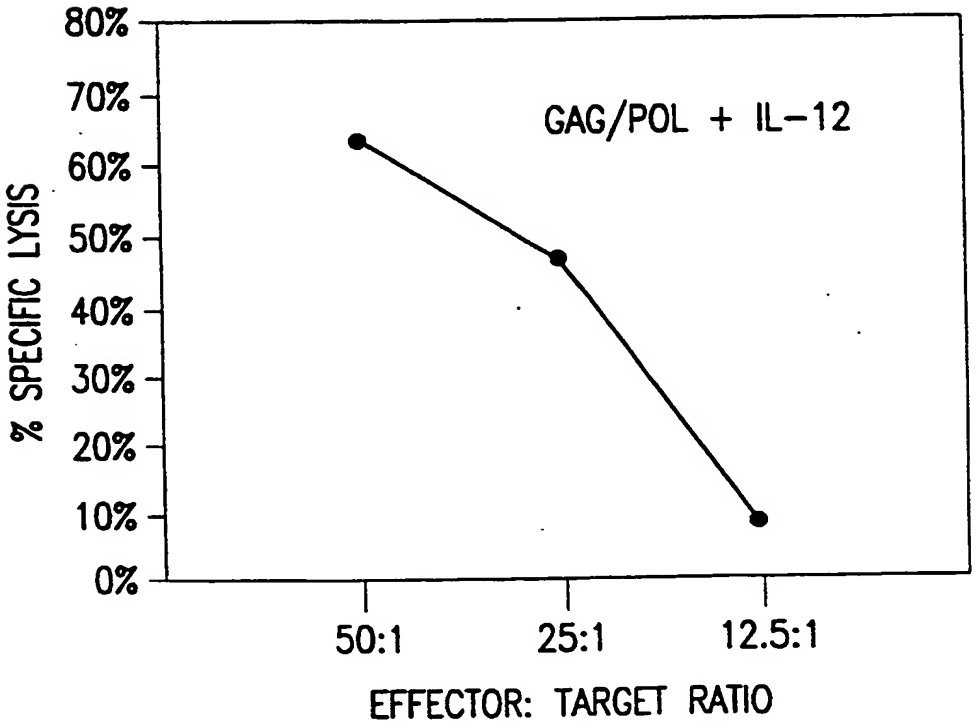


FIG.6B

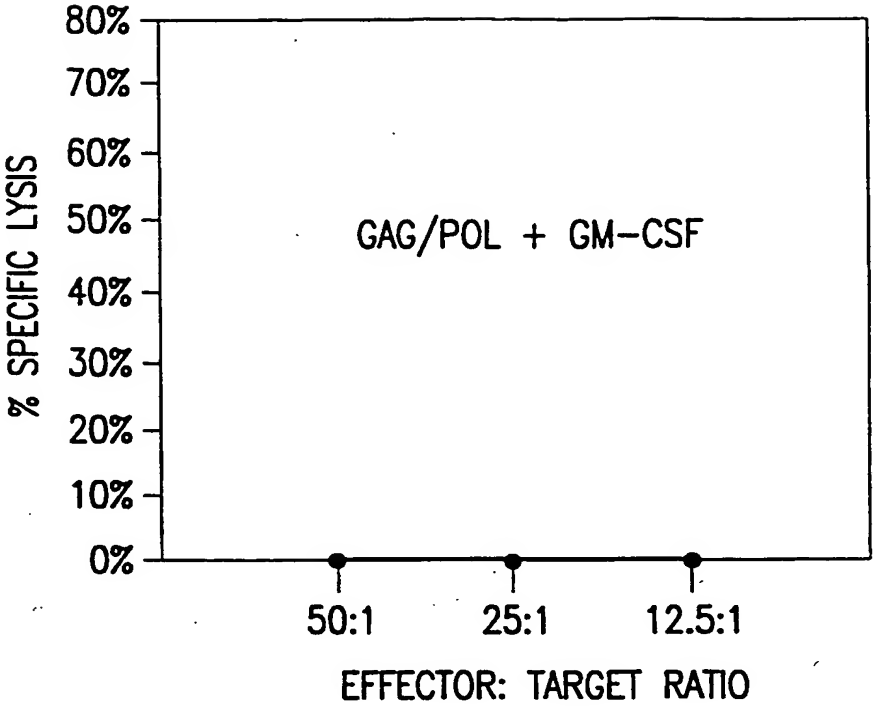


FIG.6C

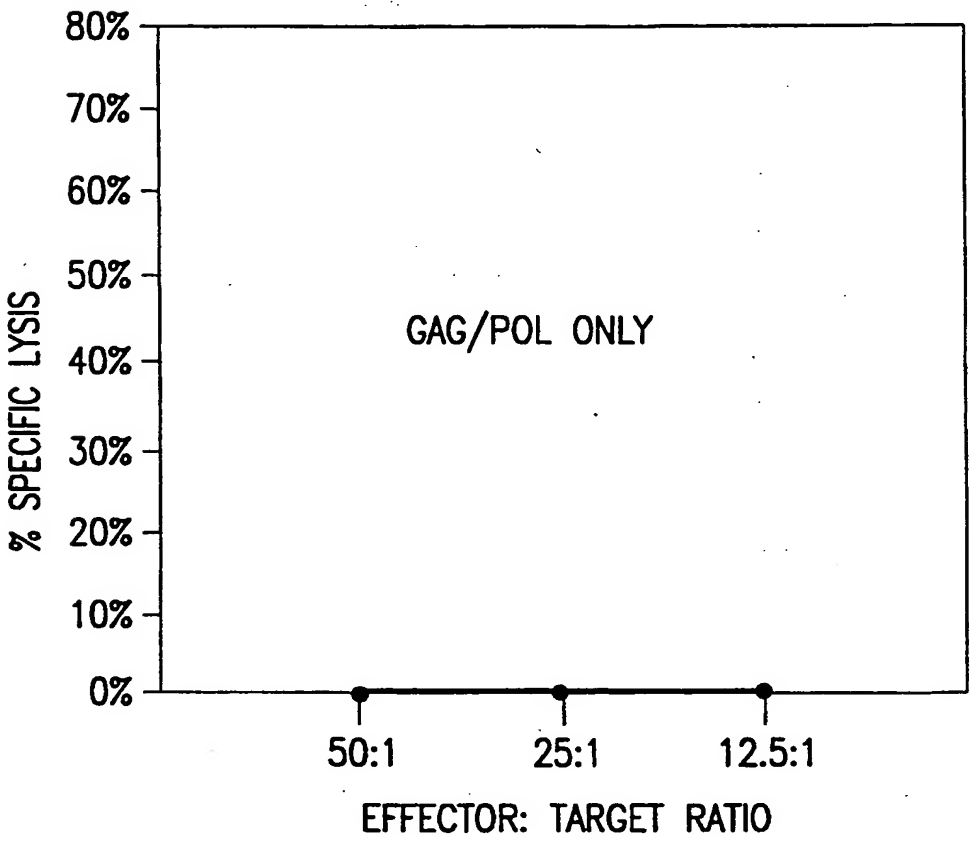


FIG.6D

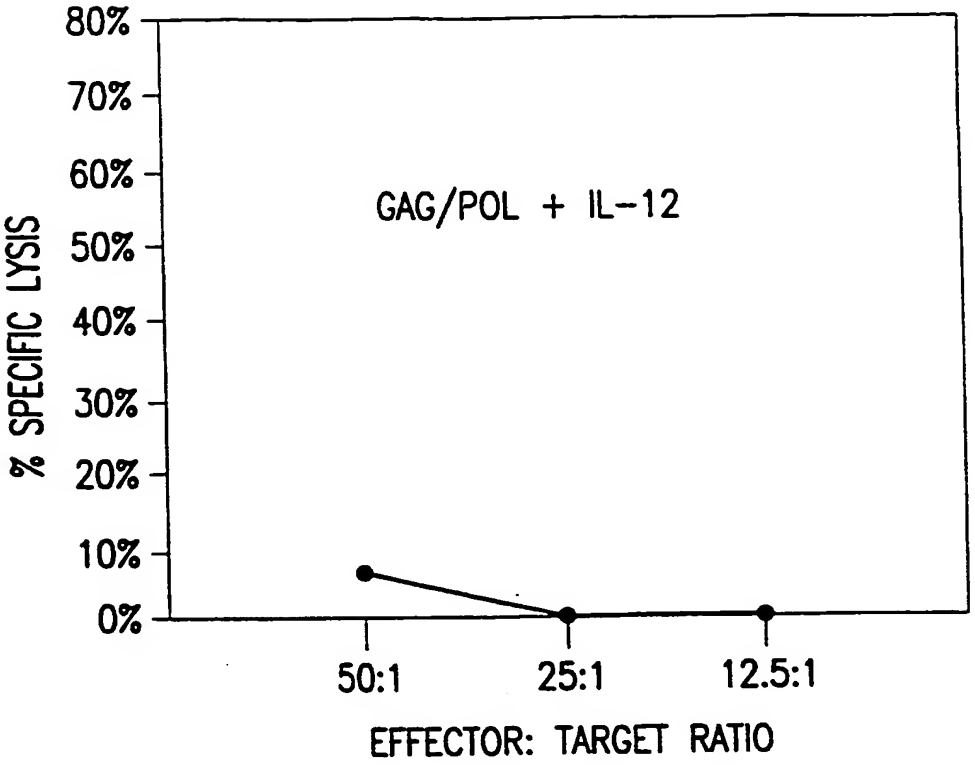


FIG.6E

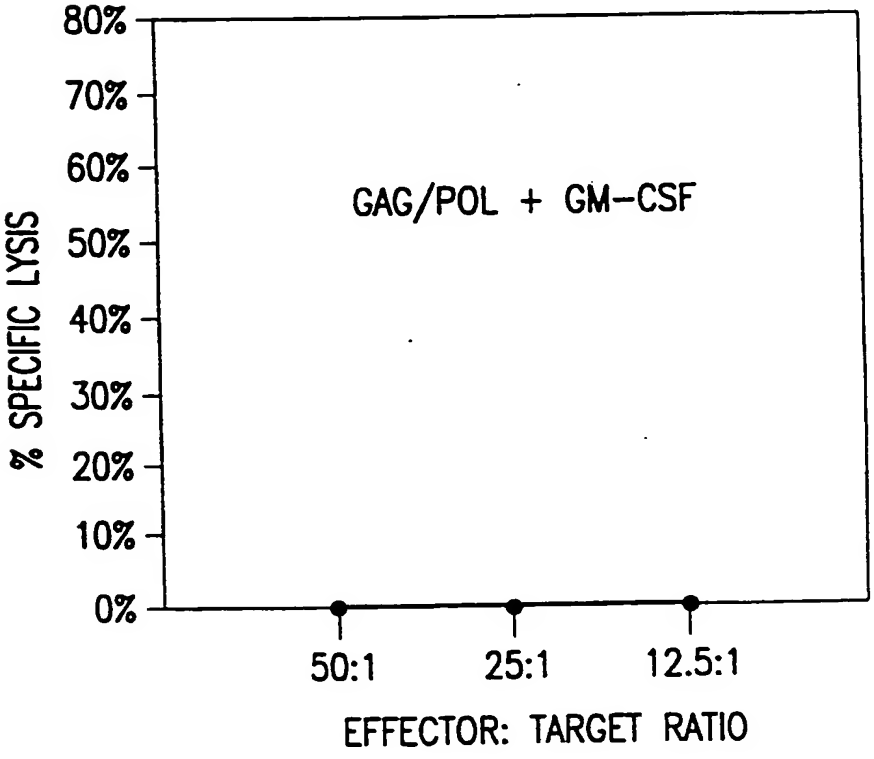


FIG.6F

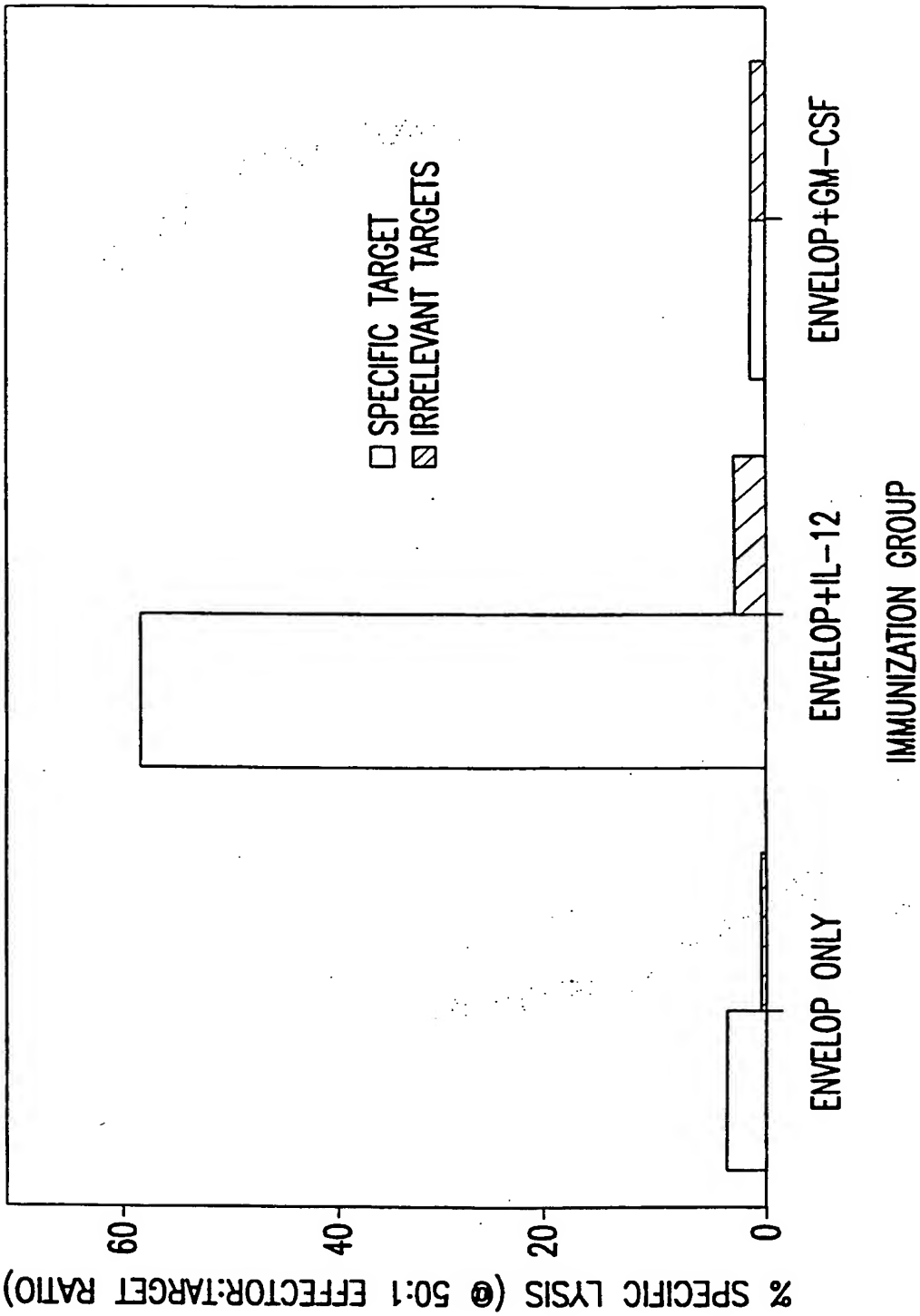


FIG.7

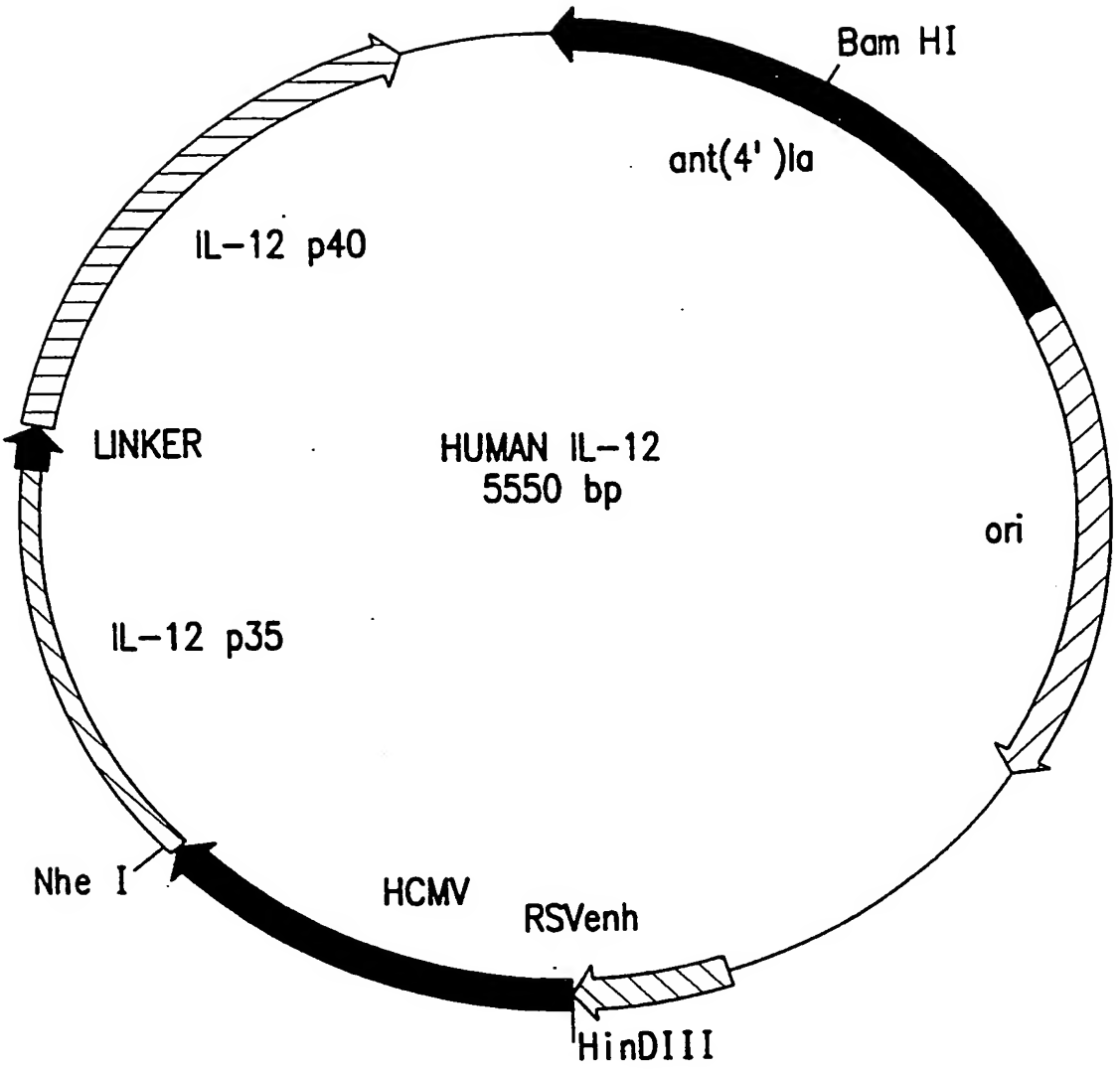


FIG.8A

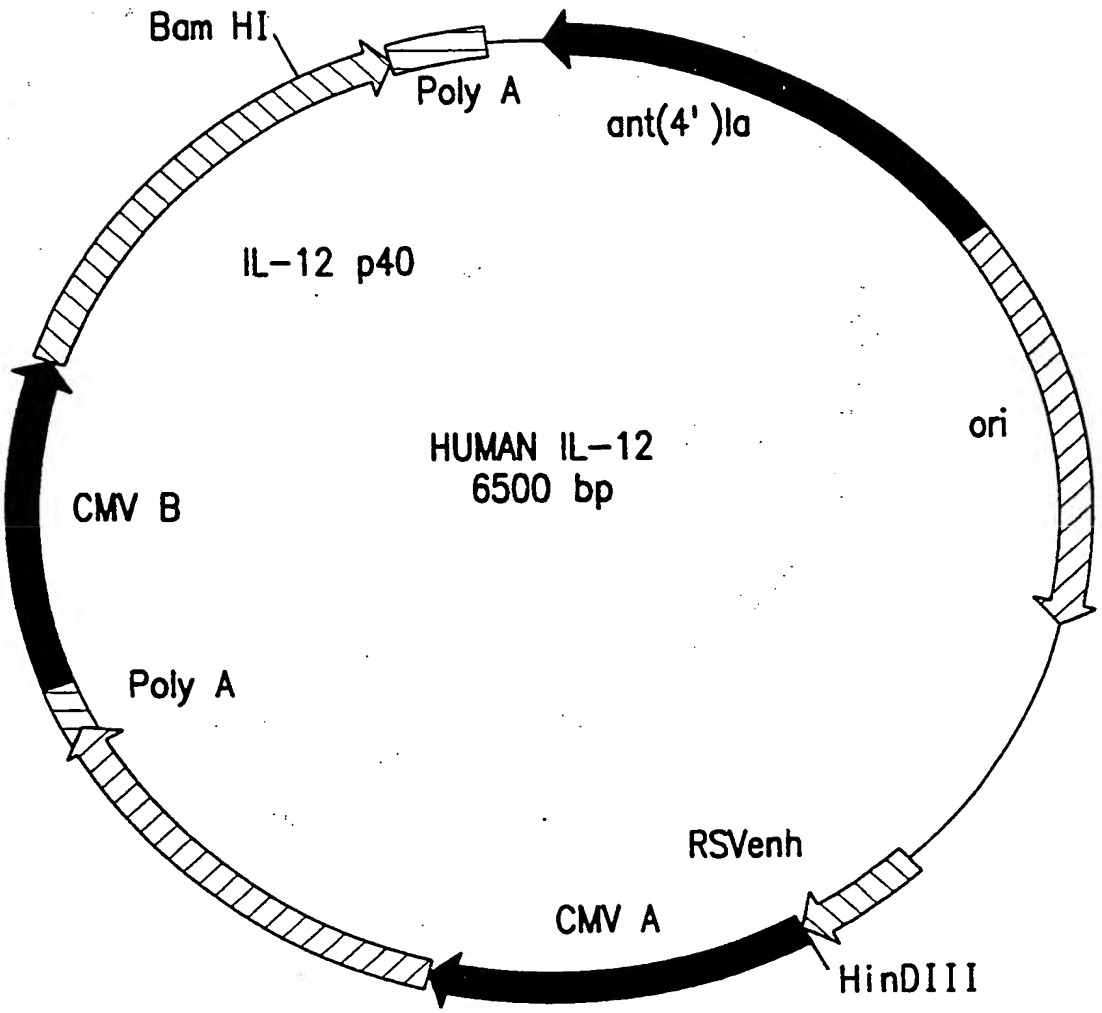


FIG.8B

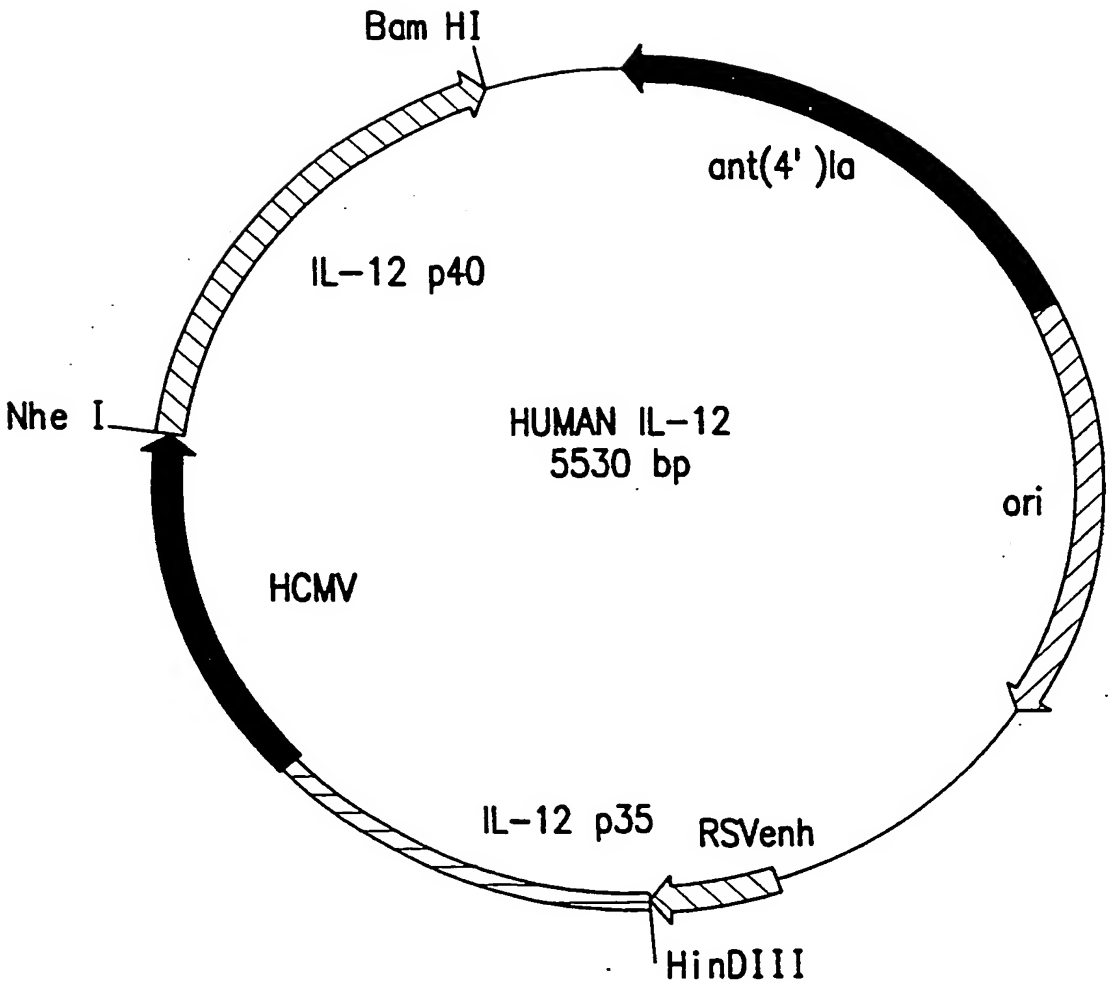


FIG.8C

% CELL POPULATION IN SPLEEN (BY FACS ANALYSIS)			
	UNIMMUNIZED	IMMUNIZED WITH Env+IL-12	IMMUNIZED WITH GAG/POL+IL-12
CD3-B220+	25.43	17.46	21.62
CD3+CD4+	34.51	39.52	32.08
CD3+CD8+	13.69	21.72	16.88

FIG.9

FIGURE 10

Construction of Cytokine Gene Expression Cassettes

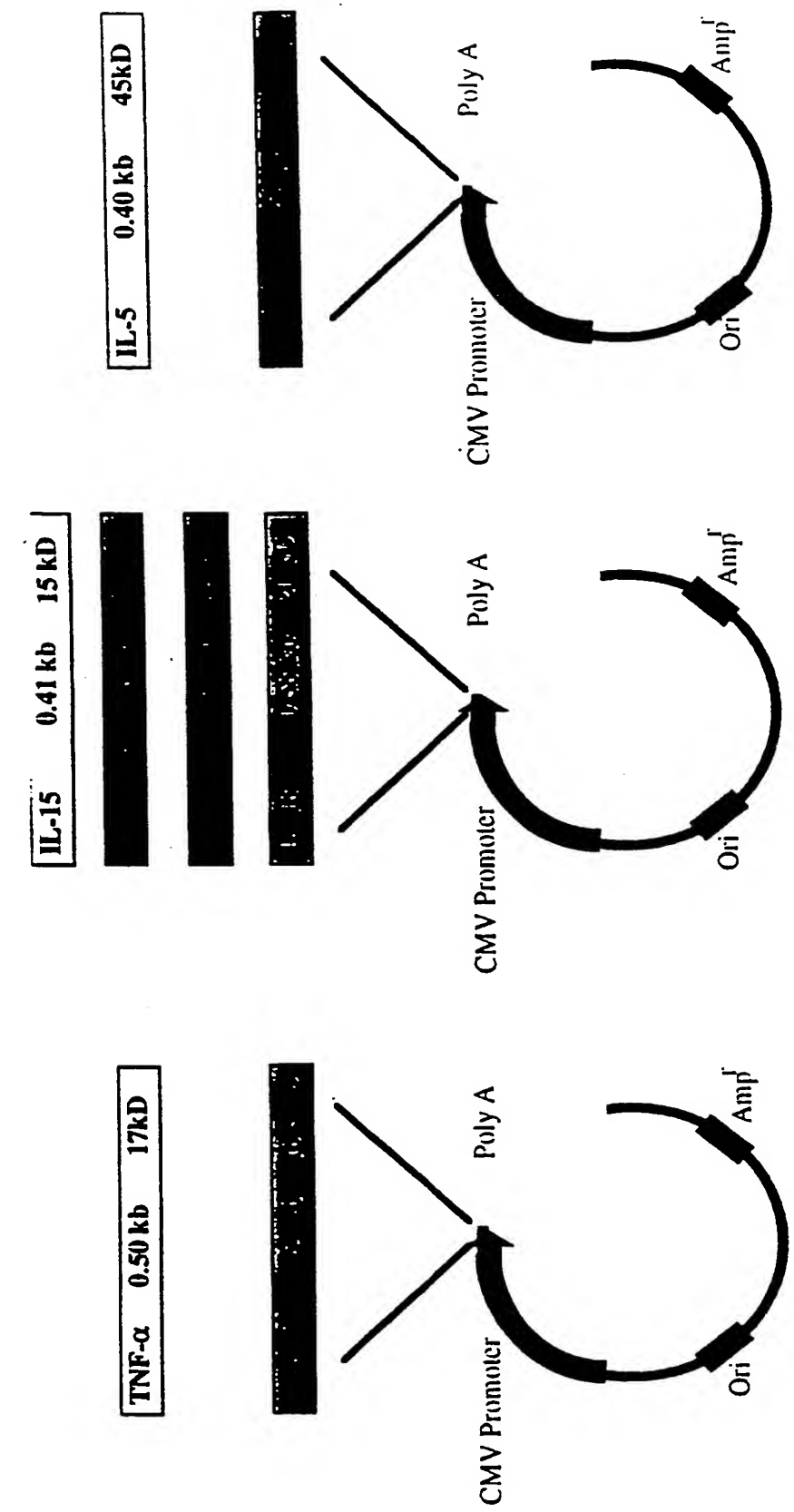


FIGURE 11A

Envelope Specific CTL Response

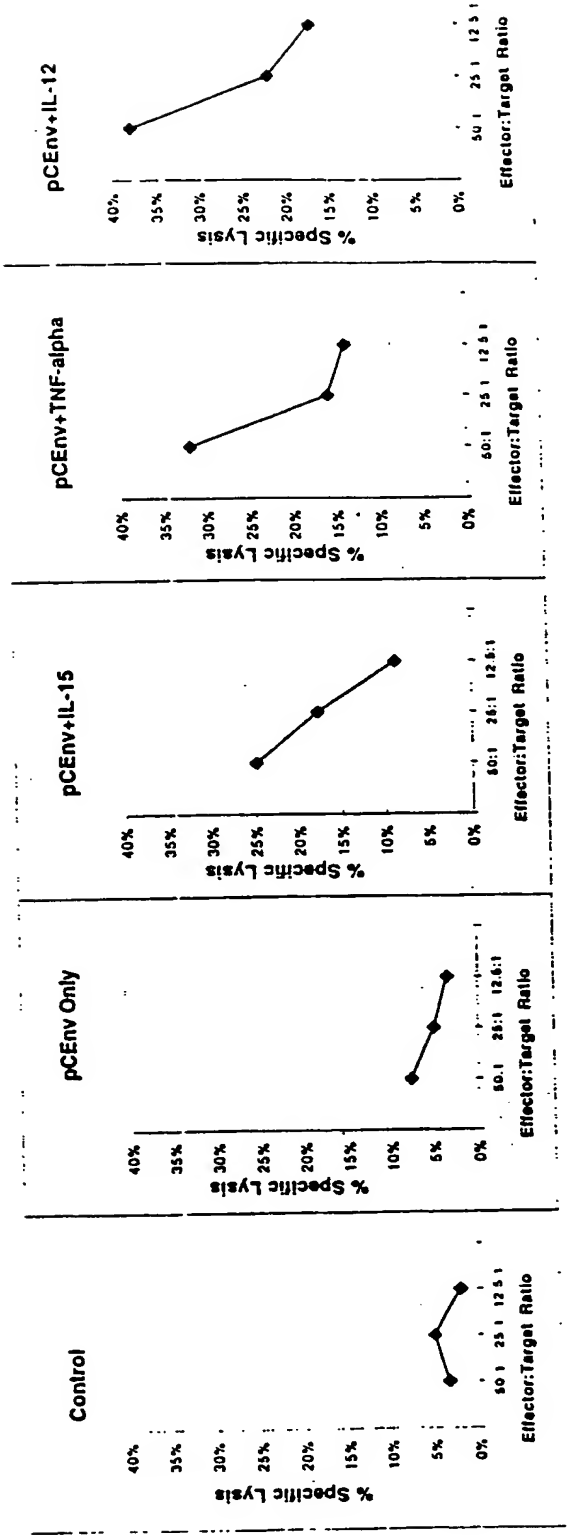
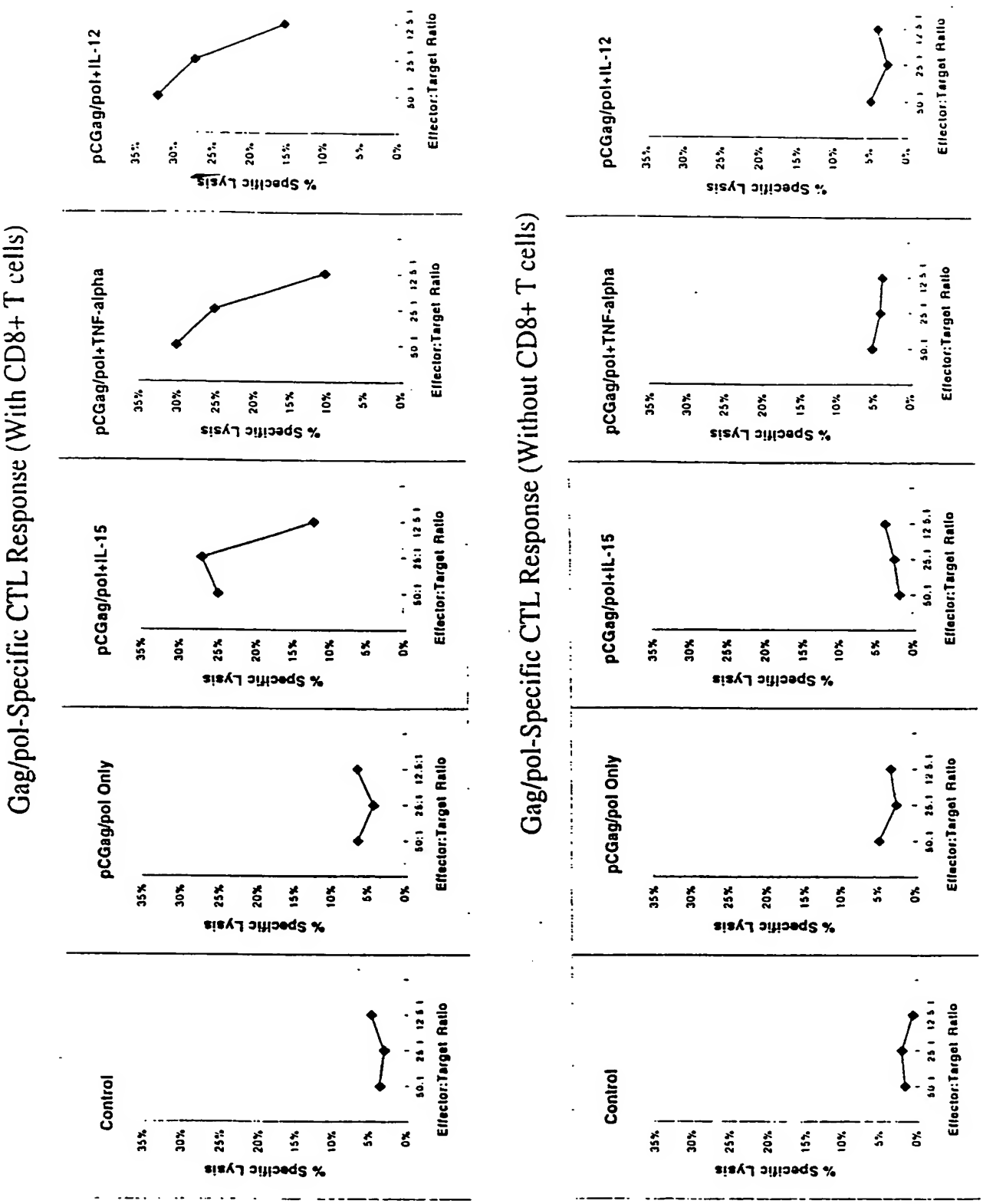


FIGURE 11B



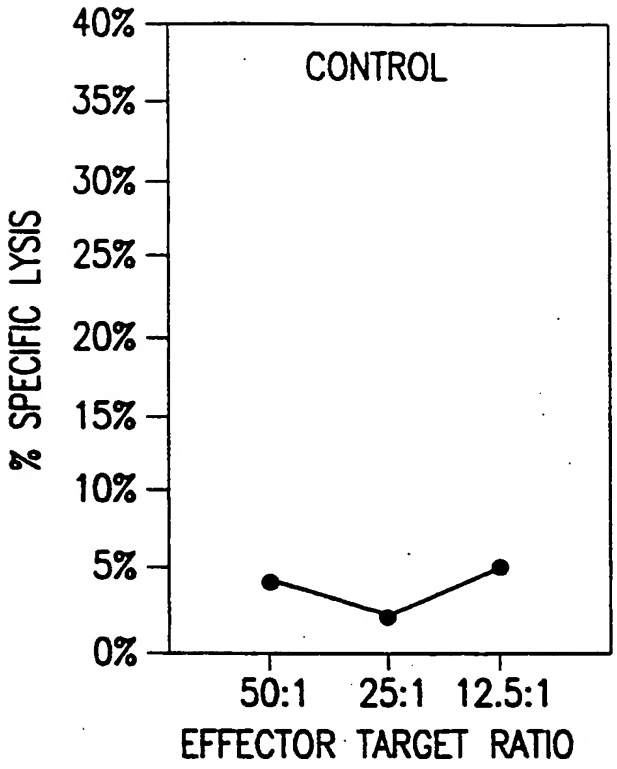


FIG.12A

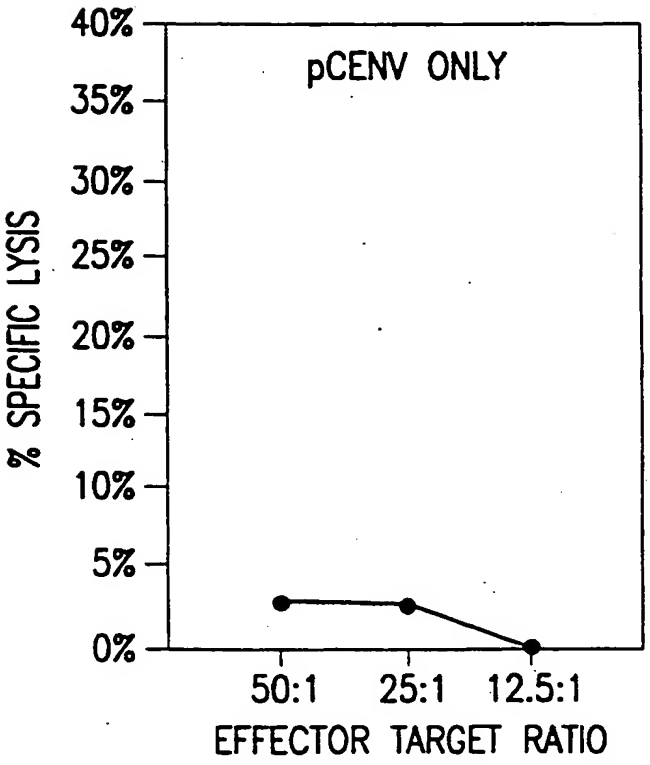


FIG.12B

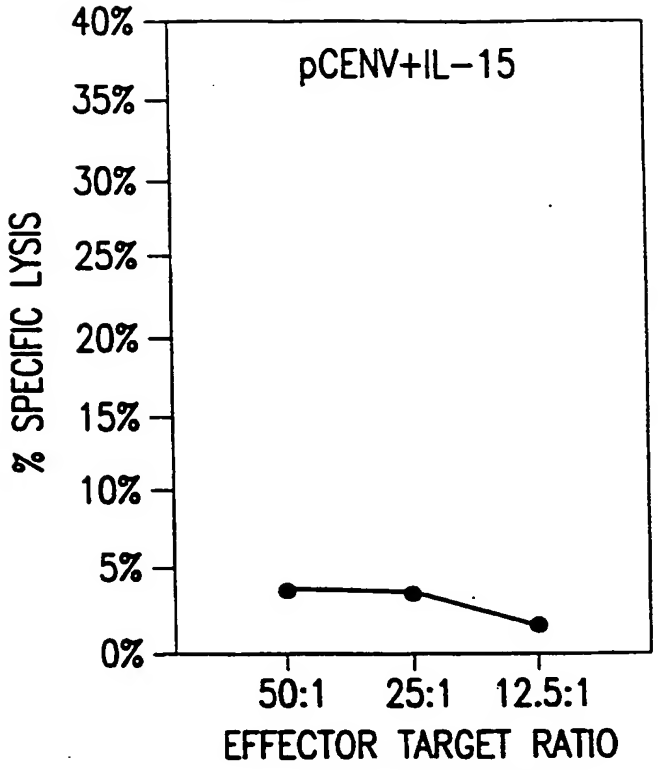


FIG.12C

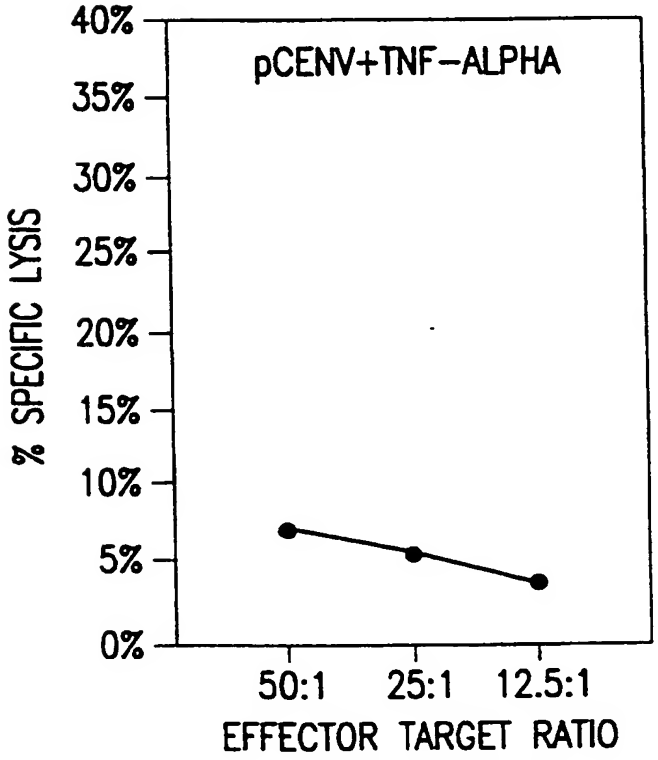


FIG.12D

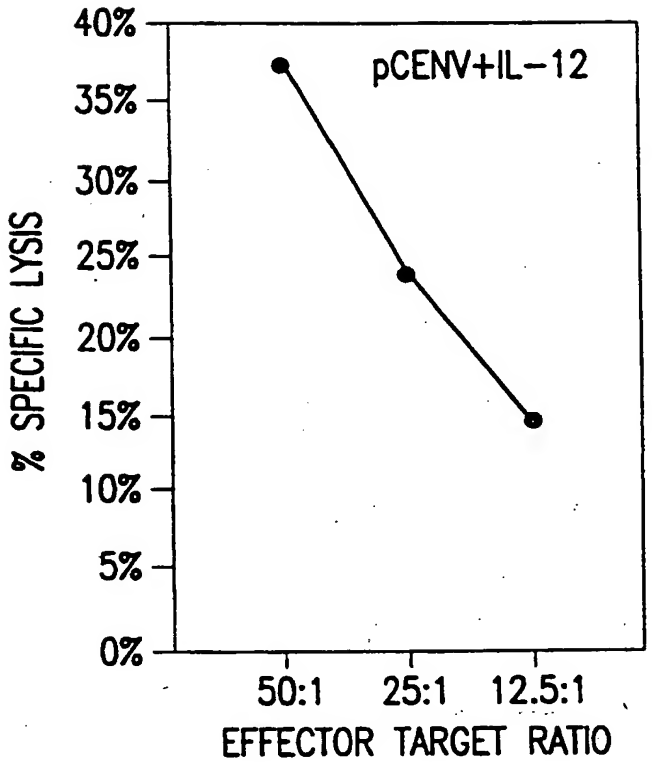


FIG.12E

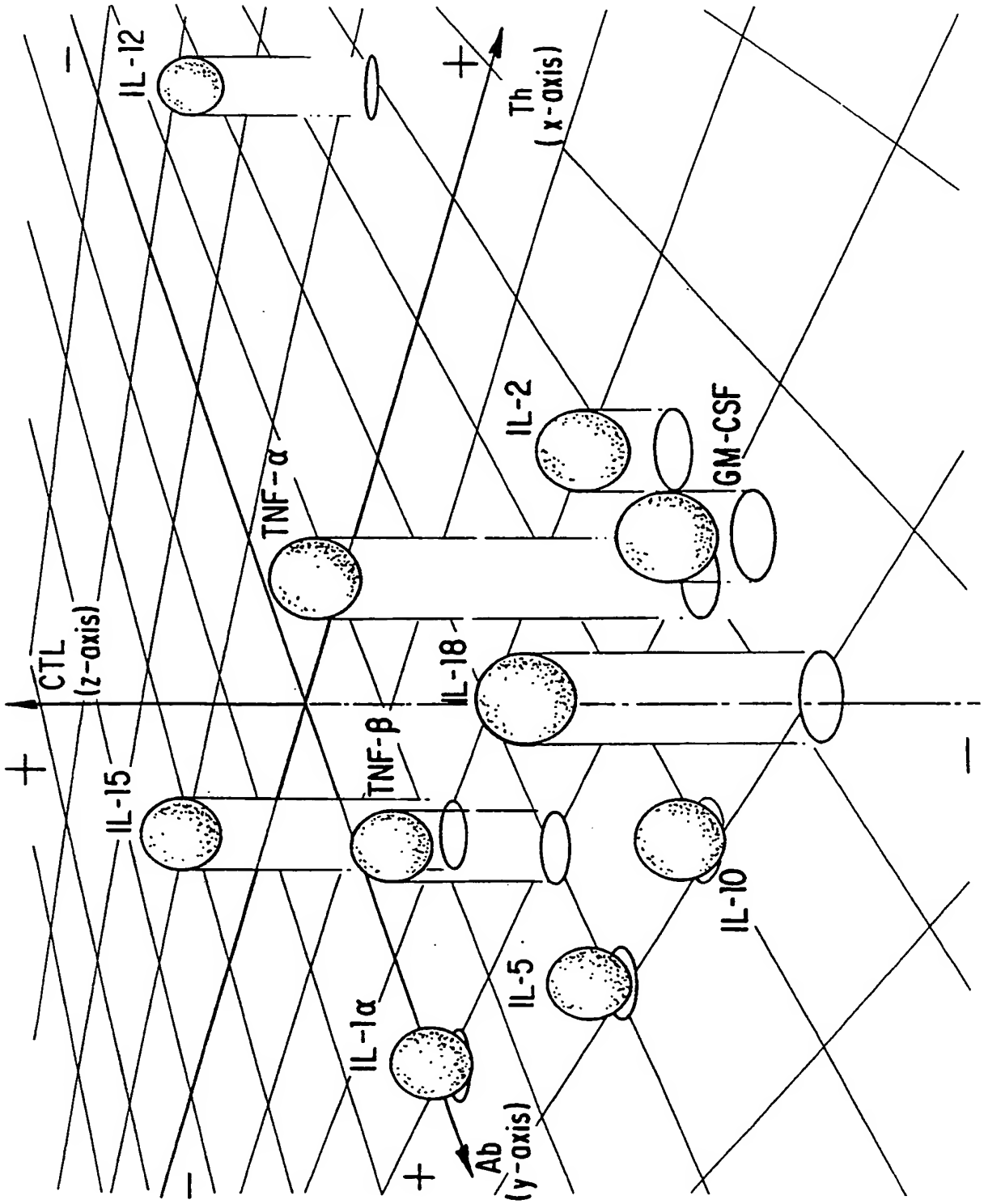


FIG.13

b7-2.ampr -> 3 phase Translation
DNA sequence 728 b.p. TTACCGAGCTCG ... CTCTGGGTACCC linear

```
1/1                                                 31/11                                                 61/21
TTA CCG AGC TCG GAT CCA CTA GTA AAC GGC CGC CAG TGT GCT GGA ATT CGG CTT AAC TCG AGC CAA AAT GGA TCC CCA GTG CTG CAA TGA
L  P  S  S  D  P  L  V  N  G  R  Q  C  A  G  I  R  L  N  S  S  Q  N  G  S  P  V  L  Q  *
 Y  R  A  R  I  H  *  *  T  A  A  S  V  L  E  F  G  L  T  R  A  K  M  D  P  Q  C  C  N  D
  T  E  L  G  S  T  S  K  R  P  P  V  C  W  N  S  A  *  L  E  P  K  W  I  P  S  A  A  M  I
91/31                                               121/41                                                151/51
TAC CGC GAG ACC CAC GCT CAC CGG CTC CAG ATT TAT CAG CAA TAA ACC AGC CAG CCG GAA GGG CCG AGC GCA GAA GTG GTC CTG CAA CTT
Y  R  E  T  H  A  H  R  L  Q  I  Y  Q  Q  *  T  S  Q  P  E  G  P  S  A  E  V  V  L  Q  L
 T  A  R  P  T  L  T  G  S  R  F  I  S  N  K  P  A  S  R  K  G  R  A  Q  K  W  S  C  N  F
  P  R  D  P  R  S  P  A  P  D  L  S  A  I  N  Q  P  A  G  R  A  E  R  R  S  G  P  A  T  L
181/61                                              211/71                                                241/81
TAT CCG CCT CCA TCC AGT CTA TTA ATT GTT GCC GGG AAG CTA GAG TAA GTA GTT CGC CAG TTA ATA GTT TGC GCA ACG TTG TTG CCA TTG
Y  P  P  P  S  S  L  L  I  V  A  G  K  L  E  *  V  V  R  Q  L  I  V  C  A  T  L  L  P  L
 I  R  L  H  P  V  Y  *  L  L  P  G  S  *  S  K  *  F  A  S  *  *  F  A  Q  R  C  C  H  C
  S  A  S  I  Q  S  I  N  C  C  R  E  A  R  V  S  S  S  P  V  N  S  L  R  N  V  V  A  I  A
271/91                                              301/101                                               331/111
CTA CAG GCA TCG TGG TGT CAC GCT CGT CGT TTG GTA TGG CTA CAT TCA GCT CCG GTT CCC AAC GAT CAA GGC GAG TTA CGT GAT CCC CCA
L  Q  A  S  W  C  H  A  R  R  L  V  W  L  H  S  A  P  V  P  N  D  Q  G  E  L  R  D  P  P
 Y  R  H  R  G  V  T  L  V  V  W  Y  G  Y  I  Q  L  R  F  P  T  I  K  A  S  Y  V  I  P  H
  T  G  I  V  V  S  R  S  S  F  G  M  A  T  F  S  S  G  S  Q  R  S  R  R  V  T  *  S  P  M
361/121                                             391/131                                               421/141
TGT TGT GCA AAA AAG CGG TTA GCT CCT TCG GTC CTC CGA TCG TTG TCA GAA GTA AGA TGG CCG CGT GTT ATC ACT CAT GGT TAT GGC AGC
C  C  A  K  K  R  L  A  P  S  V  L  R  S  L  S  E  V  R  W  P  R  V  I  T  H  G  Y  G  S
 V  V  Q  K  S  G  *  L  L  R  S  S  D  R  C  Q  K  *  D  G  R  V  L  S  L  M  V  M  A  A
  L  C  K  K  A  V  S  S  F  G  P  P  I  V  V  R  S  K  M  A  A  C  Y  H  S  W  L  W  Q  H
```

FIG.14A

```
451/151                                 481/161                                 511/171
ACT GCA TAA TTC TCT TAC TGT CAT GCC ATC CGC AAG ATG CTT TTC TGT GAC TGG TGA GTA CTC AAC CAA GTC ATT CCC GAA ATA TGT ATG
T   A   *   F   S   Y   C   H   A   I   R   K   M   L   F   C   D   W   *   V   L   N   Q   V   I   P   E   I   C   M
 L   H   N   S   L   T   V   M   P   S   A   R   C   F   S   V   T   G   E   Y   S   T   K   S   F   P   K   Y   V   C
  C   I   I   L   L   L   S   C   H   P   Q   D   A   F   L   *   L   V   S   T   Q   P   S   H   S   R   N   M   Y   A
541/181                                 571/191                                 601/201
CGC GAC CGA GTT GCT CTT TGC CCG GCG TCA ATA CGG GAT AAT CCG CGC CCC TAC CAA ACT TTC AAA AGT GCT CTC ATT GGG AAA ACT TCT
R   D   R   V   A   L   C   P   A   S   I   R   D   N   P   R   P   Y   Q   T   F   K   S   A   L   I   G   K   T   S
 A   T   E   L   L   F   A   R   R   Q   Y   G   I   I   R   A   P   T   K   L   S   K   V   L   S   L   G   K   L   L
  R   P   S   C   S   L   P   G   V   N   T   G   *   S   A   P   L   P   N   F   Q   K   C   S   H   W   E   N   F   F
631/211                                 661/221                                 691/231
TCC GGG GCG AAA ACT CCC AGA TCT TTA CCG TTG AAA TCA TTT CAT TAC CCA TCT CTC CAA CTG ATT CAC CTC TTT ATT TTC CCA CCC TCT
S   G   A   K   T   P   R   S   L   P   L   K   S   F   H   Y   P   S   L   Q   L   I   H   L   F   I   F   P   P   S
 P   G   R   K   L   P   D   L   Y   R   *   N   H   F   I   T   H   L   S   N   *   F   T   S   L   F   S   H   P   L
  R   G   E   N   S   Q   I   F   T   V   E   I   I   S   L   P   I   S   P   T   D   S   P   L   Y   F   P   T   L   W
721/241
GGG TAC CC
G   Y
 G   T
  V   P
```

FIG.14B

mber	2	Name	APL 400-004
te	1/22/96	Made By	Apollon, Inc. One Great Valley Pkwy Malvern, PA 19355-1423
ctor	APL-400-004		
seri	None	Comments	Alias pBB-Kana. New version.
esistance	KANA		
ector Size	4000 bp		
sert Size	None		
ombined Size	4000		

ab Location

Map

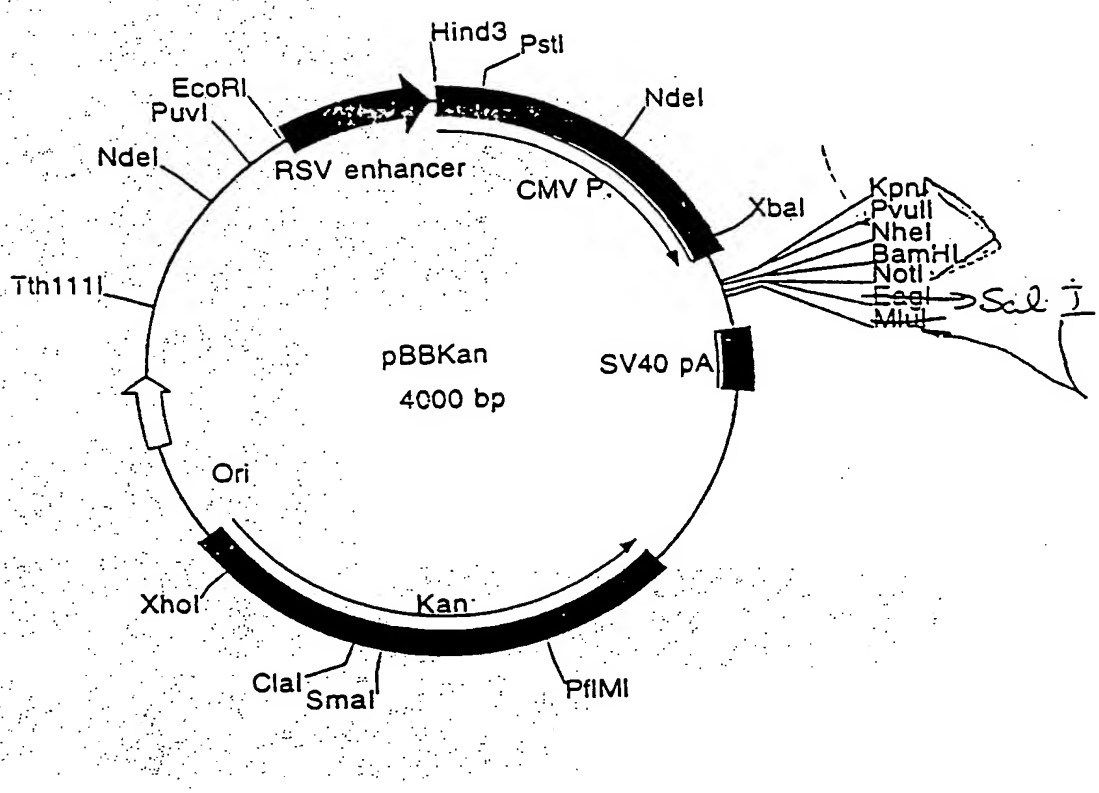


FIGURE 15

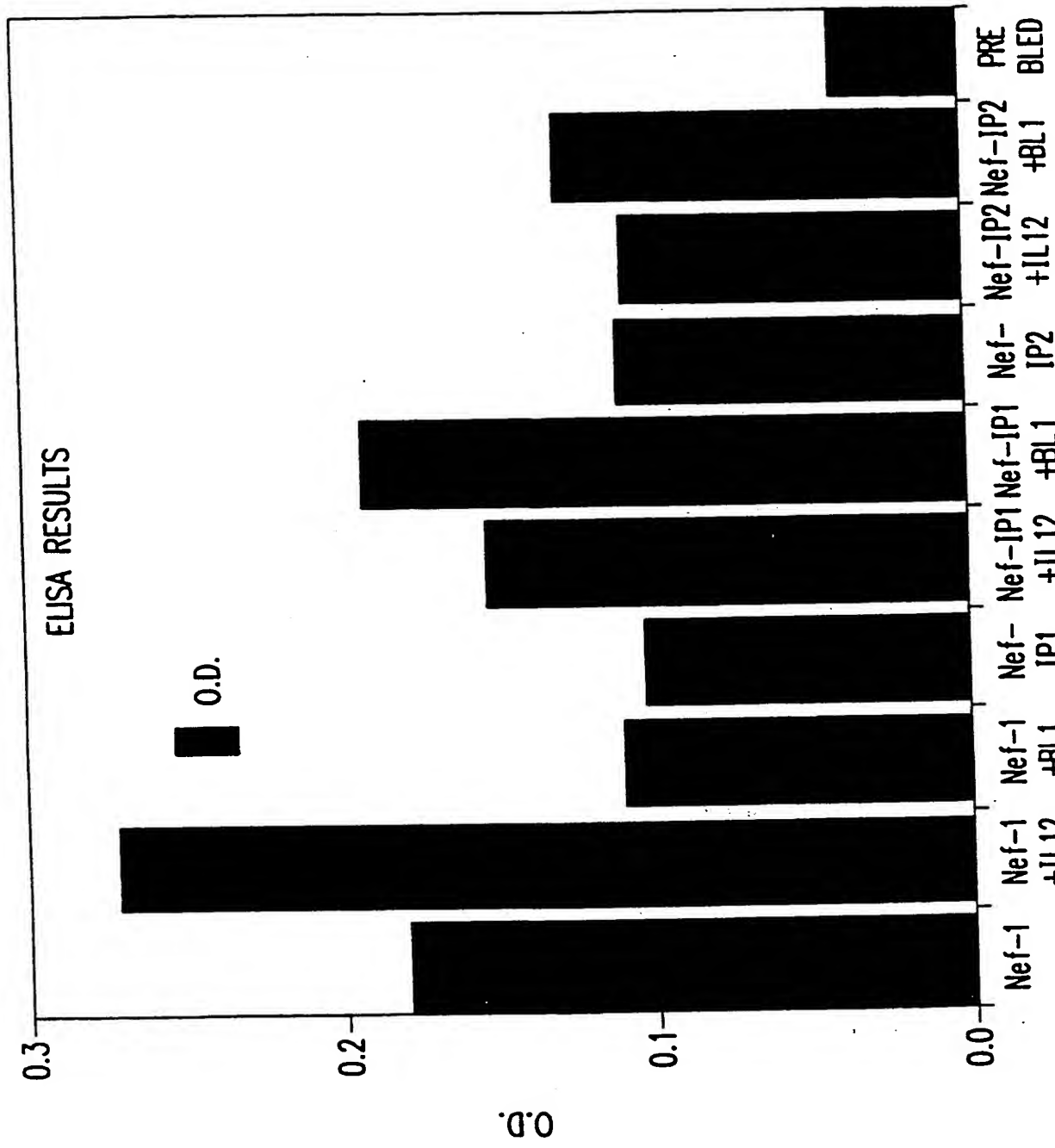


FIG.16

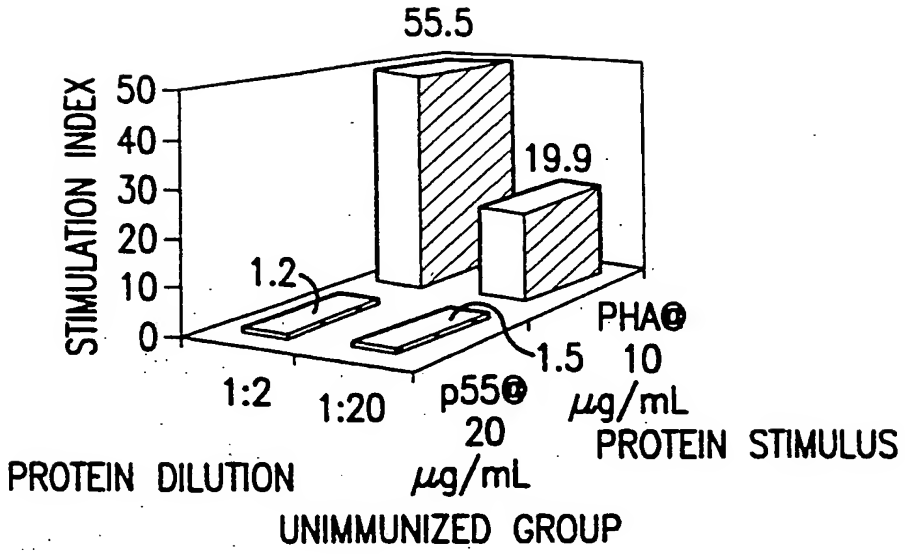


FIG.17A

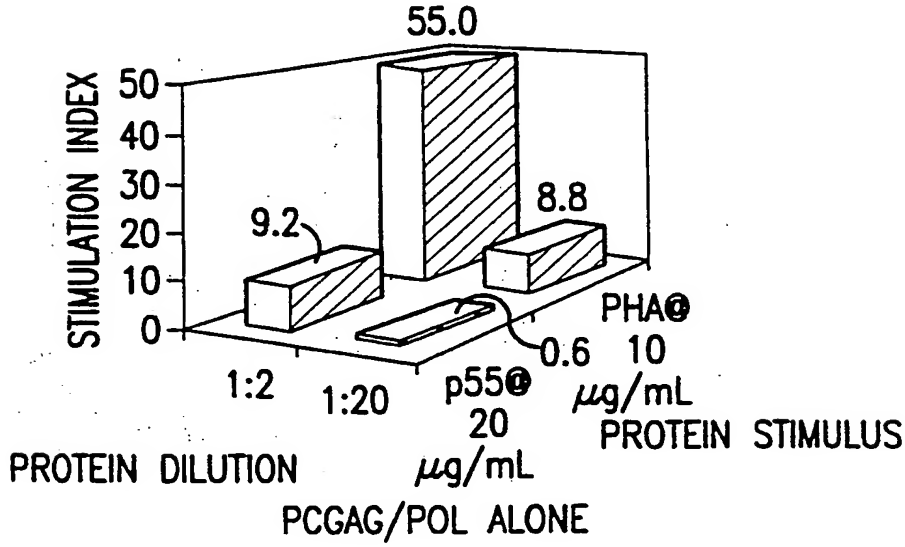
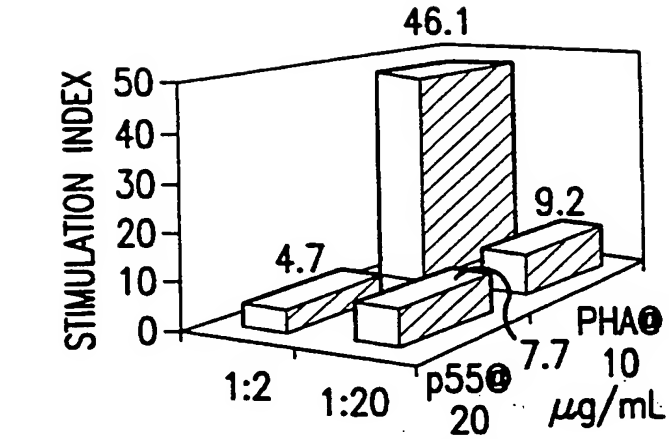
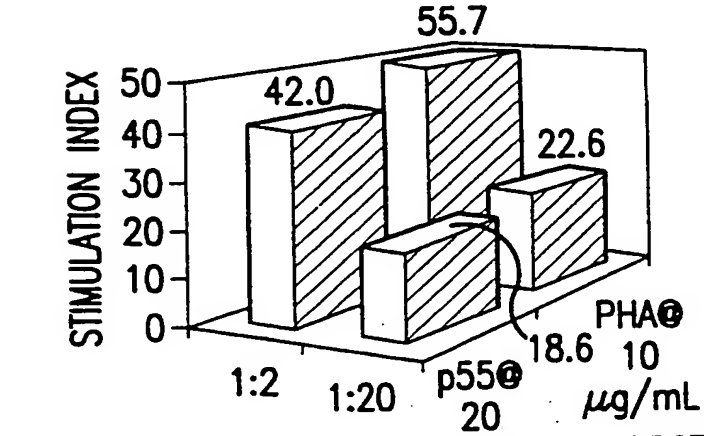


FIG.17B



PCGAG/POL+CD80 CO-IMMUNIZATION

FIG.17C



PCGAG/POL+BL-1 CO-IMMUNIZATION

FIG.17D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19502

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 15/18; A61K 48/00, 31/00
US CL :514/44; 424/278.1; 435/320.1, 172.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/278.1; 435/320.1, 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS, CAPLUS
search terms: DNA, plasmid, immunogen, immunization, cytokine, vaccine.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,238,823 A (POTTER ET AL) 24 August 1993 (24.08.93), see entire document, especially columns 15-16.	1-2, 18-19
X -- Y	WO 88/00971 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATON) 11 February 1988 (11.02.88), pages 9-10.	1-3, 5-8, 18-20, 22-29 --- 9-11, 13-17
A	US 5,457,038 A (TRINCHIERI ET AL) 10 October 1995 (10.10.95), see entire document, especially columns 21-22.	4, 12, 21, 30
X -- Y	WO 95/24485 A2 (MERCK & CO., INC.) 14 September 1995 (14.09.95), see entire document, especially lines 1-29.	1-8, 18-29 --- 9-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
17 DECEMBER 1997

Date of mailing of the international search report
03 FEB 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
PREMA MERTZ
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19502

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-30

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19502

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-30, drawn to a plasmid comprising a nucleotide sequence that encodes an immunomodulating protein and a nucleotide sequence that encodes an immunogen, pharmaceutical compositions thereof, a method of immunizing an individual by administering the plasmid, and a pharmaceutical composition comprising 2 or more such plasmids and a method of immunizing with such.

Group II, claims 31-33 and 36, drawn to a BL-1 protein, a recombinant expression vector comprising a nucleic acid encoding such and a pharmaceutical composition comprising a nucleic acid molecule encoding BL-1 protein.

Group III, claims 34-35, drawn to an antibody which binds to an epitope of BL-1 protein.

Claims 32-36 have been interpreted as reasonably being dependent on claim 31.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Pursuant to 37 C.F.R. § 1.475(d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of each of the other categories related thereto. Accordingly, the main invention (Group I) comprises the first recited product, a plasmid comprising a nucleotide sequence that encodes an immunomodulating protein and a nucleotide sequence that encodes an immunogen, pharmaceutical compositions thereof, a method of immunizing an individual by administering the plasmid, and a pharmaceutical composition comprising 2 or more such plasmids and a method of immunizing with such. Further pursuant to 37 C.F.R. § 1.475(d), the ISA/US considers that any feature which the subsequently recited products and methods share with the main invention does not constitute a special technical feature within the meaning of PCT Rule 13.2 and that each of such products and methods accordingly defines a separate invention.